Mesenchymal Stromal Cells for graft-versus-host disease

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Mesenchymal Stromal Cells for graft-versus-host disease

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To Alina and our little Lorenzo,

my inspiration and support

Abstract

The immunosuppressive activity of Mesenchymal Stromal Cells (MSCs) is well documented, but the therapeutic benefit is completely unpredictable. Prospective randomized clinical trials remain the only means to address MSC clinical efficacy. However, their success is undermined by the difficulty to stratify patients. Probably the most challenging conundrum is that, despite being immunosuppressive, MSCs are undetectable following administration. Therefore, understanding the fate of infused MSCs could help to shed light on the mechanisms of MSC immunobiology and predict clinical responses.

We decided to focus our attention on MSCs used for the treatment of Graft versus Host Disease (GvHD), since there is proof-of-principle of their efficacy. In order to address the mechanisms used by MSCs to deliver immunosuppression we adopted a preclinical model of GvHD by which we demonstrated that MSCs are actively induced to undergo perforin-dependent apoptosis by recipient cytotoxic cells and that this process is essential to initiate MSC-induced immunosuppression. When examining patients with GvHD who received MSCs we found a striking parallel, whereby only those with high cytotoxic activity against MSCs responded to MSC infusion whereas those with low activity did not. Importantly, the need for recipient cytotoxic cell activity could be replaced by the infusion of apoptotic MSCs generated *ex vivo*. After infusion, recipient phagocytes engulfed apoptotic MSCs and produce indoleamine 2,3-dioxygenase (IDO) that is ultimately necessary for effecting immunosuppression.

These results point out that crucial events for the delivery of MSC immunosuppressive activity take place soon after infusion. This observation was also supported by the retrospective analysis of the clinical data of a cohort of 60 steroid-resistant acute GvHD patients treated with MSCs, since the assessment of the response as early as 1 week after MSC administration is sufficient for predicting the survival of the patients.

In summary, we propose the innovative concept that response should be assessed early after starting MSC treatments, and patients should be stratified for MSC treatment according to their ability to kill MSCs. Our results strongly suggest the intriguing possibility to treat patients with immune disorders with *ex vivo* apoptotic MSCs.

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List of Abbreviations

1-DMT:	1-methyl-D-tryptophan
1-MT:	1-Methyltryptophan
aGvHD:	Acute Graft versus Host Disease
ALL:	Acute Lymphoblastic Leukemia
AML:	Acute Myeloid Leukemia
APC:	Antigen Presenting Cells
ApoMSCs:	MSCs made apoptotic in vitro
AT:	Adipose tissue
ATG:	Anti-thymocyte globulin
BAL:	Bronchoalveolar lavage
BM:	Bone Marrow
CAf:	Caspase activity
CCR:	C-C receptor
cDCs:	Classical Dendritic Cells
cGvHD:	Chronic Graft versus Host Disease
CML:	Chronic Myeloid Leukemia
ConA:	Concanavalin A
COX:	Cyclooxygenase

CR: Complete response

CSA: Cyclosporine

CTLA-4: Cytotoxic T-Lymphocyte–associated Antigen-4

CXCR: C-X-C chemokine receptor

DC: Dendritic Cells

ECP: Extracorporeal photopheresis

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene-bis(oxyethylenenitrilo)tetraacetic acid

FOXP3: Forkhead box P3

FRET: Förster Resonance Energy Transfer

FRET-MSCs: MSCs transfected with pECFP-DEVDR-Venus

GrB: Granzyme B

GvHD: Graft versus Host Disease

HC: Healthy controls

hIFNγ: Human Interferon-γ

HL: Hodgkin Lymphoma

HLA: Human Leucocyte Antigen

HO-1: Heme oxygenase-1

HPRT1: Hypoxanthine phosphoribosyltransferase 1

HSC: Haematopoietic Stem Cells

HSCT:	Haematopoietic Stem Cell Transplantation
hTNFα:	Human TNF-α
i.p.:	Intraperitoneally
i.v.:	Intravenously
IBD:	Inflammatory bowel disease
ICAM:	Intercellular Adhesion Molecule 1
IDO:	Indoleamine 2,3-dioxygenase
IFNγ:	Interferon-γ
Ig:	Immunoglobulin
IL:	Interleukin
iTreg:	Induced Regulatory T cells
JAK1/2:	Janus kinase 1/2
LPS:	Lipopolysaccharide
Luc-MSCs:	MSCs transfected with luciferase
MDS/MPNs:	Myelodysplastic Syndrome/Myeloproliferative Neoplasms
MEP:	Methylprednisolone
Mh:	Matahari
MHC:	Major Histocompatibility Complex
mIFNγ:	Murine Interferon-γ

MLR: Mixed Lymphocyte Reactions

MLR-aPBMCs: Pre-activated with MLR

MM: Multiple Myeloma

MMF: Mycophenolate Mofetil

MPS: Mononuclear Phagocyte System

MSC: Mesenchymal Stromal Cells

mSpl: Murine splenocytes

mTNFα: Murine TNF-α

MTX: Methotrexate

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NHL: Non-Hodgkin Lymphoma

NK: Natural Killer

Nre: Not Reported

NR: Non-Responders

ns: Not significant

nTreg: Natural Regulatory T cells

NY-ESO-1: New York Oesophageal Squamous Cell Carcinoma-1

OS: Overall survival

OVA: Ovalbumin

PBMC: Peripheral Blood Mononuclear Cells

PCR: Polymerase chain reaction

PD-1: Programmed death-1

PD-1: Programmed death-2

pDCs: Plasmacytoid Dendritic Cells

PGE2: Prostaglandin E2

PHA: Phytohemagglutinin

PHA-aPBMCs: PBMCs pre-activated with PHA

PKCζ-PS: MYR Protein Kinase-Cζ Pseudosubstrate

PR: Partial response

PTSG2: Prostaglandin-Endoperoxide Synthase 2

R: Responders

RNA: Ribonucleic acid

RPMI: Roswell Park Memorial Institute medium

TCR: T-cell receptor

TGF-β: Transforming growth factor-β

Th: T helper

Th3: TGF-β-secreting T helper 3 cells

TLR: Toll-like receptor

TLS:	Total luminescence signal
TNFα:	Tumour Necrosis Factor-α
Tr1:	T regulatory type 1
TRAIL:	TNF-related apoptosis-inducing ligand
Treg:	Regulatory T cells
TSG-6:	Tumour necrosis factor-inducible gene 6
UC:	Umbilical cord
VCAM:	Vascular cell adhesion molecule 1
VLA-4:	Very late antigen-4
Z-AAD-CMK:	Methyl 5-chloro-4-oxo-3-[2-[2 (phenylmethoxycarbonylamino) propanoylamino [propanoylamino] pentanoate
Z-ApoMSCs:	ApoMSCs obtained adding the pan-caspase inhibitor Z-VAD-FMK during the 24 hours of incubation
Z-VAD-FMK:	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl] fluoromethylketone

List of publications accepted during the PhD period

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2) **Galleu A**, Milojkovic D, Deplano S, Szydlo R, Loaiza S, Wynn R, Marks D.I, Richardson D, Orchard K, Kanfer E, Tholouli E, Saif M, Sivaprakasam P, Lawson S, Bloor A, Pagliuca A, Potter V, Mehra V, Snowden J.A, Vora A, Kishore B, Hunter H, Apperley J.F, and Dazzi F. Mesenchymal Stromal Cells for acute Graft versus Host Disease: response at one week predicts probability of survival. Accepted in British Journal of Haematology, 11 December 2018.

3) Trento C, Bernardo ME, Nagler A, Kuçi S, Bornhäuser M, Köhl U, Strunk D, **Galleu A**, Sanchez-Guijo F, Gaipa G, Introna M, Bukauskas A, Le Blanc K, Apperley J, Roelofs H, Van Campenhout A, Beguin Y, Kuball J, Lazzari L, Avanzini MA, Fibbe W, Chabannon C, Bonini C, Dazzi F. Manufacturing mesenchymal stromal cells for the treatment of graft-versus-host disease: a survey amongst centers affiliated to the European Group of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant*. 2018 Jul 19. pii: S1083-8791(18)30402-6. doi: 10.1016/j.bbmt.2018.07.015.

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7) Sweeney NP, Regan C, Liu J, **Galleu A**, Dazzi F, Lindemann D, Rupar CA, McClure MO. Rapid and efficient stable gene transfer to mesenchymal stromal cells using a modified foamy virus vector. *Mol Ther*. 2016 May 2. doi: 10.1038/mt.2016.91

Major Achievements

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2018	Runner up for the best poster at the Postgraduate Research Symposium, granted by School of Cancer & Pharmaceutical Sciences, King's College London, London.
2018	Jon J. van Rood Award, For the Best Paper in the Immunology of Allogeneic Hematopoietic Transplantation in the Cellular Therapy and Immunobiology, granted by the scientific committee of the European Society of Blood and Marrow Transplantation and Cellular Therapy & Immunobiology Working Party (EBMT-CTIWP).

1 General Introduction

1.1 The mesenchymal stromal cells dilemma: one, hundreds or thousands of entities?

Mesenchymal Stromal Cells (MSCs) consist of a highly heterogeneous population of stem and progenitor cells selected and expanded *in vitro* as unfractionated fibroblastic-like and plastic-adherent cells[1]. A precise definition of these cells yet remains predominantly ambiguous, despite the surge of references in the literature and the escalation of the number of clinical trials using these cells during the last decades[2]. The MSC acronym itself, widely employed and acknowledged from the scientific community, has been challenged in relation to its real connotation.

First isolated in mouse bone marrow in the late sixties, as non-haematopoietic, adherent cells able to give rise to colonies of fibroblastic-shaped cells (thus defined as colony-forming units–fibroblastic)[3], these cells were then termed “marrow stromal stem cells” by Owen twenty years later[4], based on their origin (*marrow*), their property of self-renewal and to differentiate (*stem*) *in vitro* into a spectrum of fully differentiated cells of the connective tissue (*stromal*)[5]. In a provocative attempt to challenge the widely accepted idea that the only stem cells in mammalian organisms were haematopoietic stem cells (HSCs), Caplan coined the term “mesenchymal

stem cells" to further reinforce the notion of stemness of these cells which were for the first time referred to as "MSCs"[6]. However, the capacity of MSCs to differentiate into different lineages was initially confined to *in vitro* experiments[7], thus making the actual relevance of the stemness property equivocal. It was then proposed to use the more conservative expression, multipotent mesenchymal stromal cells[8], and minimal criteria for defining MSCs[9] were introduced. Based on these criteria, MSCs were defined by the positivity of few non-specific markers (CD105, CD73 and CD90) and negativity of others (CD14 or CD11b, CD19, CD31, CD34, CD45, CD79a), combined with the capacity to produce polyanionic, hydrophobic and mineralized products under specific and artificial *in vitro* conditions[9]. Cells meeting these criteria could be obtainable from any (virtual all) stromal tissues[10–15] and could be extremely variable in regards to differentiation stage, proliferation rates, functional characteristics and morphology, as already demonstrated by early investigations[4,7,16,17]. Although new sets of markers have been recently proposed, the characterization of MSCs yet remains elusive[18]. Main obstacles to a better clarification originate by the extreme difficulty in understanding whether MSC heterogeneity originates from the different culture manipulations or reflects the innate heterogeneity of the *in vivo* repertoire of MSC subsets[19]. To this regard, many investigations have been performed to try to identify the cell/s of MSC origin in the body. Since its very first isolation, MSCs were hypothesized as part of a wide stromal cell system of the body[4]. It has now become apparent that most MSCs derive from specific progenitors located mainly in the perivascular space[20], thus posing the question of the identity between MSCs and pericytes[21].

However, this concept has been challenged by the observation that MSCs are not located exclusively around vessels[22] and not always MSCs and pericytes share the same markers[23].

Despite their wide heterogeneity, MSCs have been used as cellular therapeutic tools in many diseases. The successes of early studies, whereby MSCs were used to sustain engraftment after Haematopoietic Stem Cell Transplantation (HSCT)[24], or to ameliorate disparate diseases such as Osteogenesis Imperfecta[25] or Graft versus Host Disease (GvHD)[26], greatly contributed to the confidence on MSC potentials. Clear evidence of this climate is the incredible fast-paced increase of studies whereby MSCs were used for the treatment of diseases affecting multiple sites[27–31].

Two main MSC features underpin the therapeutic potentials of MSCs: regenerative and immunosuppressive properties. They are not mutually exclusive or completely independent as tissue regeneration requires resolution of injury-associated inflammation.

The multipotency of MSCs will not be further discussed (reviewed in [32] and [20]). Conversely, their immunosuppressive properties will be extensively examined since they represent the main topic of this dissertation.

1.2 Immunomodulating properties of MSCs

1.2.1 General concepts in MSC immunobiology.

Among MSC functions not related to stemness or multipotentiality, the most intriguing is their immunomodulatory activity. There is extensive evidence that MSCs exert potent immunosuppressive effects *in vitro* against virtually any cell of both the innate and adaptive immune system (Figure 1.1). Despite the degree of MSC heterogeneity described above, this property seems to be shared with many (if not all) stromal cells from different tissues[33–37]. However, since Bone Marrow (BM) derived MSCs were the first to be isolated and tested in patients, they still remain the most commonly used MSCs in clinical studies and represent the term of comparison for MSCs obtained from other tissues.

$CD4^+$ and $CD8^+$ T cells are both significantly inhibited by MSCs in a non-specific and non-selective way, regardless of their previous encounter with their cognate antigen[38], activation state or type of T-cell receptor (TCR) expressed[39]. Both allogeneic and autologous MSCs can exert immunosuppression, thus suggesting that immunomodulation is not mediated through major histocompatibility complex (MHC) restriction[40,41]. In the presence of MSCs, T-cell proliferation is reversibly inhibited[41] and their cell cycle is arrested in the G0/G1 phase through inhibition of cyclin D2 and up-regulation of p27Kip1 expression[42]. This effect does not seem to interfere with

T cell activation processes, since the expression of CD69, CD25, and cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) on T cells is similar in the presence or absence of MSCs[43]. Despite this very potent MSC anti-proliferative activity, also contrasting results have been reported. Indeed, while at lower Peripheral Blood Mononuclear Cells (PBMC)/MSC ratios MSCs exerted potent anti-proliferative activity against PBMCs, at higher ratios this property was reverted and PBMC proliferation indeed stimulated[44]. Similarly, the addition of poly(I:C) (Toll-like receptor 3 [TLR3] ligand) and Lipopolysaccharides (TLR4 ligand) to the T cell/MSC co-culture almost completely abrogated MSC anti-proliferative activity[45]. Taken together, these observations seem to suggest that the MSC suppressive activity is dependent on culture conditions and their ratio with T cells.

As for T cells, MSC effects on Natural Killer (NK) cells seem to be complex and strictly dependent on the balance between environmental cues and activation state of NK cells. It has been demonstrated that MSCs could inhibit freshly isolated or resting NK cell proliferation upon stimulation with interleukin-2 (IL-2) alone[46], IL-2 in combination with T-cell depleted allogeneic PBMCs[43], or IL-15[47]. This inhibition was associated with significant reduction in NK production of the inflammatory cytokines Interferon-γ (IFN-γ) and Tumour Necrosis Factor-α (TNF-α), and in NK cytotoxic activity against their targets[43,47]. Conversely, NK cell proliferation is only minimally affected by the presence of MSCs when NK cells are pre-activated with IL-2[46]. In this setting, both autologous and allogeneic MSCs were lysed by activated NK cells and the cytotoxic activity seems to be mediated by the MSC expression of the

proteins UL16 binding protein, poliovirus receptor (CD155) and Nectin-2, ligands of the NK activating molecules natural killer group two C and DNAX Accessory Molecule-1, respectively[46]. Similar results were obtained when NK cells were pre-activated with IL-15[47]. Notably, when MSCs were pre-treated with IFN-γ before the co-culture with IL-2 activated NK cells, their susceptibility to lysis was completely reverted as a result of up-regulation of Human Leucocyte Antigen (HLA) type I on MSC surface[46].

MSCs exert their immunomodulatory effects also against B cells. Several studies using murine *in vitro* models demonstrated the anti-proliferative activity of MSCs when in co-culture with B cells[42], with decrease in the IgG secretion and expression of the activation marker CD25[48] through the involvement of the Programmed Death-1 (PD-1) pathway[49]. Similar findings were obtained using co-culture of human B cells and MSCs. In this system, B cells were arrested in the G0/G1 phase of the cell cycle as T cells, while their viability was preserved[50]. Furthermore, the production of IgM, IgG, and IgA was inhibited and chemotaxis through C-X-C chemokine receptor-4 (CXCR4) and CXCR5 axes impaired[50]. Nevertheless, these results were in contrast with other reports whereby MSCs had no effect on B cell proliferation[43], or conversely had a stimulatory effect on B cell proliferation and secretory activity[51]. As observed for T and NK cells, also the MSC immunomodulatory activity on B cells seems to be dependent on environmental cues.

Taken together, these observations underpin one of the most important features of MSC immunobiology: its functional plasticity. MSC inhibitory activity is not constitutive, but it needs to be elicited by the inflammatory microenvironment through a mechanism commonly defined as “licensing”[1]. Cytokines mainly involved in this process are IFN-γ[43,52], IL-1β and TNF-α[53]. Nonetheless, the licensing effect on MSCs could change, depending on the specific inflammatory stimuli. Indeed, it has been observed that specific concentration of IFN-γ could turn MSCs into potent Antigen Presenting Cells (APCs)[54–56], while the presence of TLR3 and TLR4 ligands could impair the MSC immunosuppressive properties and restore an effective T cell response[57].

As described in this paragraph, MSCs exert a broad arsenal of immunosuppressive capabilities which place them as important coordinator of the immune system. This multifactorial role cannot be executed only by a direct activity on the ultimate immune effector cells but it requires also additional contact-dependent and -independent signals with other regulatory cells[1,33,58]. It has been proposed that it is through either a direct and an indirect activity that MSCs can create a network of cellular interactions and communication which eventually define the impact of MSCs on inflammatory responses. In the next two paragraphs, these two not mutually exclusive mechanisms will be discussed.

Figure 1.1. Summary of the immunosuppressive mechanisms employed by MSCs described *in vitro*.

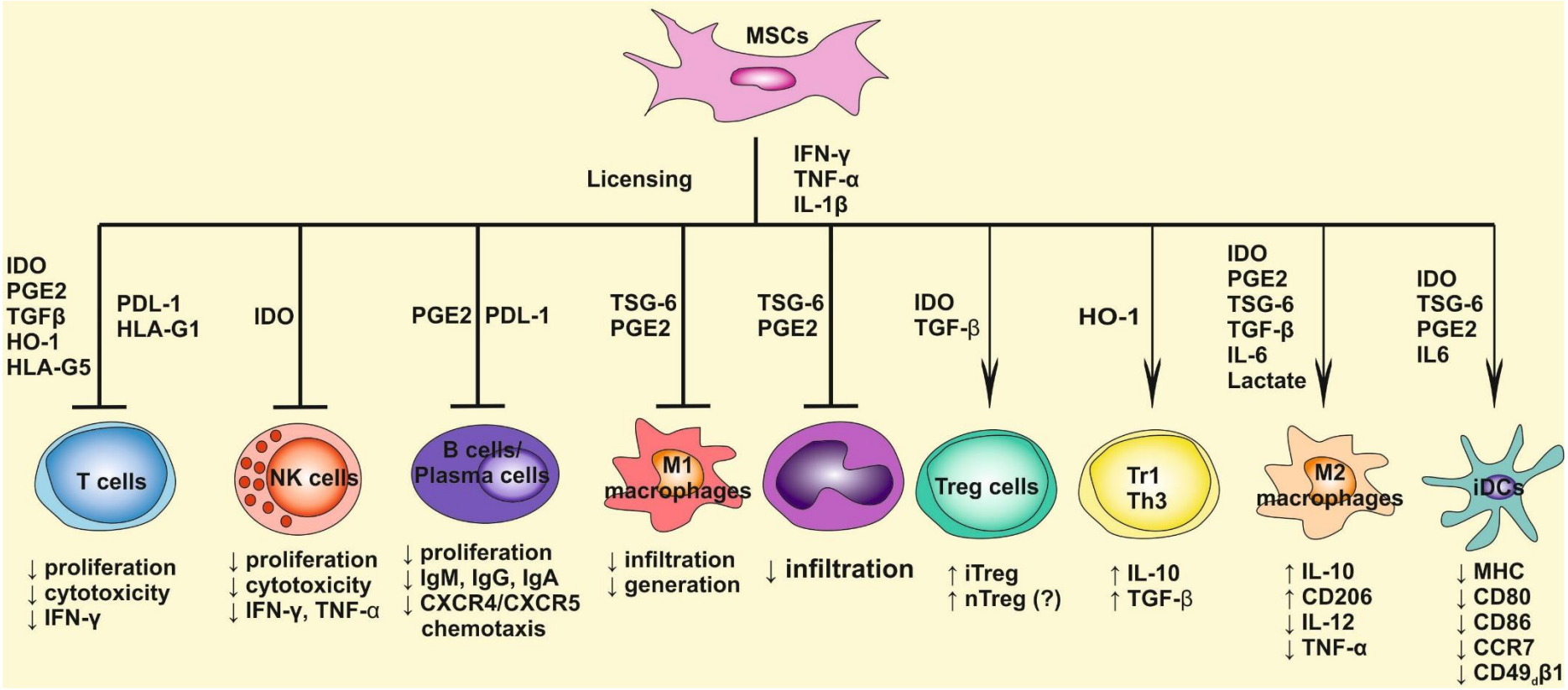

Figure 1.1. Summary of the immunosuppressive mechanisms employed by MSCs described *in vitro*. In the presence of an inflammatory environment, MSCs are “licensed” to produce different molecules which can mediate a very potent immunosuppressive activity against any cell of the immune system. Blunt line: inhibition. Arrowed line: stimulation. **CCR7**: C-C receptor-7. **CXCR4**: C-X-C chemokine receptor type 4. **CXCR5**: C-X-C chemokine receptor type 5. **HLA**: human leukocyte antigen. **HO-1**: heme oxygenase-1. **IDO**: Indoleamine 2,3-dioxygenase. **IFNγ**: Interferon. **IgA**: Immunoglobulin A, **IgG**: Immunoglobulin G, **IgM**: Immunoglobulin M. **IL-1β**: Interleukin-1β. **IL-6**: Interleukin-6. **IL-10**: Interleukin. **IL-12**: Interleukin -12. **iTreg**: induced Regulatory T cells. **MHC**: major histocompatibility complex. **nTreg**: natural Regulatory T cells. **PGE2**: Prostaglandin E2. **PDL-1**: Programmed death-ligand 1. **TSG-6**: Tumour necrosis factor-inducible gene 6. **TGF-β**: Transforming growth factor-β

1.2.2 MSC immunosuppression by direct activity

The secretion of soluble factors plays a fundamental role in MSC immunosuppression [33], and several factors have been described.

1.2.2.1 Indoleamine 2-3 dioxygenase 1 (IDO1)

IDO1 represents the best characterized enzyme in MSCs of human orgin[59]. IDO-1 is one of the rate-limiting enzymes regulating the kynurenine pathway. By controlling the oxidative cleavage of the indole ring of Tryptophan into N'-formylkynurenine, it controls the catabolism of tryptophan and its metabolites kynurenines[60]. IDO expression is tightly regulated and dependent on the presence of T helper type 1 (Th1) inflammatory cytokines (IFN-γ being the most potent inducer) in the microenvironment[61–63], or on the interaction of CTLA-4 and CpG oligonucleotides with B7 molecules[64–66] and TLR9[67], respectively. The activation of the kynurenine pathway plays a key function in both the innate and the adaptive immune systems by directly controlling pathogen replication in several chronic infections[68–71], and limiting indiscriminate damage in inflamed sites during microbial invasion[72]. However, the discovery that IDO could serve as immunosuppressive factor to protect foetal allografts from immunological rejection[73] sparked interest in investigating IDO in immune tolerance. IDO central role in the induction of tolerance to self-antigens has been comprehensively confirmed in several *in vivo* studies of allograft rejection, and autoimmune diseases (reviewed in [61]).

IDO is also important in the generation of peripheral tolerance to neoantigens expressed in tumour tissues[74]. Indeed, the use of the IDO inhibitor 1-Methyltryptophan (1-MT) was able to reverse this immunosuppression and could complement the therapeutic activity of antineoplastic drugs in eradicating experimental models of cancer. The synergistic activity of 1-MT was immune mediated, since it was lost in mice deficient of a T cell response[75]. The biochemical pathways employed by IDO to modulate immune responses are still object of investigations. What is emerging is a complex scenario whereby both tryptophan depletion and accumulation of kynurenines might play dual and not mutually exclusive roles in the generation of immunosuppressive effects. Tryptophan depletion leads to a direct suppression of T cell activation and proliferation secondary to amino acid starvation which activates the amino acid sensitive general-control-non-repressible 2 stress kinase pathway and blockage of the mammalian target of rapamycin signalling[61]. Increase of kynurenines in the microenvironment mediates the acquisition of a tolerogenic phenotype in APCs dependent on the downregulation of the expression of MHC class II and the co-stimulatory molecules CD80, CD86 and CD40[76]. Furthermore, there have been reports demonstrating that T cells are sensitive to the antiproliferative and cytotoxic activity of tryptophan metabolites themselves both *in vitro*[77] and *in vivo*[78], and that some of the kynurenines produced after tryptophan degradation could prolong skin allograft survival in rats[79]. It has been demonstrated that the generation of regulatory T cells (Treg) can be elicited by both mechanisms[80,81]. It is then plausible that more than one mechanism might be

employed by IDO in its tolerogenic activity, the relevant importance thereof varying with the specific system considered.

The production of IDO by MSCs and its role in mediation of MSC immunosuppression has been extensively described[43,59,82–85]. IDO is not constitutively expressed in MSCs, but its production is induced by the presence of IFN-γ in the microenvironment[43,59,82,84]. As described in the first part of the paragraph, also in the case of the IDO produced by MSCs, the immunosuppression can be mediated by tryptophan starvation and/or kynurenine accumulation, the importance of which varies depending on the specific setting considered[59,84]. IDO induction is also associated with the ability of MSCs to interact and orchestrate the immunosuppressive activity of other cells of the immune system such as Treg cells[85] and macrophages[83]. This important interaction will be thoroughly analysed in the paragraph entitled “MSC immunosuppression by indirect activity on regulatory cells.

1.2.2.2 Prostaglandin E2 (PGE2)

PGE2 is the most abundant eicosanoid lipid mediator in the human body and exerts a wide range of activities regulating several physio-pathological processes at disparate sites[86–88]. The synthesis of PGE2 comprises the release of the precursor arachidonic acid from the plasma membrane by phospholipase A2[89], the production of PGG2 and PGH2 by the activity of the rate-limiting enzyme Cyclooxygenase (COX), and the final conversion into

PGE2 by three specific synthases[90,91]. Two isoforms of COX have been identified: while COX1 is constitutively expressed in any tissues and mainly regulates homeostatic processes[92], COX2 can be induced by many pro-inflammatory or mitogenic stimuli (such as IL-1β, TNF-α or lipopolysaccharide [LPS])[93] and seems to account for most of the prostanoids produced during inflammation. However, this concept has been challenged by observations that also COX1 could be induced and take part into inflammatory responses in human cells *in vitro* [94,95] and in mouse systems *in vivo*[96].

PGE2 exerts its activity through ligation with four different G-protein-coupled receptors nominated as EP receptors (EP1 to EP4). Upon ligand binding, EPs specifically mediate different intracellular pathways triggering changes in the production of intracellular cyclic adenosine monophosphate, or in Ca^{2+} mobilization[97]. PGE2 is traditionally considered one of the most important mediators of the four cardinal signs of inflammation (rubor [redness], calor [heat], tumor [oedema and swelling], and dolor [pain]) [97], however its role in hampering T cell proliferation was described over 40 years ago[98].

The inhibition of T cell proliferation and activation is mediated by an interference with the IL-2/IL-2R axis[99,100] and/or with the antigen-induced TCR activation[101,102]. Furthermore, PGE2 is able to induce the expression of the transcription factor forkhead box P3 (FOXP3) in human $CD4^{+}CD25^{-}$ cells to acquire regulatory functions[103,104]. As a consequence, T cells are skewed towards a Th2 phenotypes with reduced levels of IL-2 and IFNγ, and

increased IL-4 and IL-5 production[105]. Accordingly, PGE2 induces the production of IgG1 and IgE in B cells stimulated with IL-4 and LPS[106]. However, the regulation on B cell activity seems to depend on the maturation stage. Indeed, while enhancing the proliferation of mature B cells, PGE2 exerts anti-proliferative activity on immature B cells[107].

However, the role of PGE2 in immunomodulation is far more complex than previously thought. For example, while PGE2 stimulates DCs activation in peripheral tissues, it exhibits opposite effects after Dendritic Cells (DCs) have migrated to the lymph nodes, whereby DC maturation and antigen-presenting capacity is inhibited[93]. Furthermore, PGE2-licensed DCs induce naive T cells to differentiate into Th2 cells[108]. Conversely, if DCs are treated with PGE2 in the presence of LPS, they secrete large amounts of IL-23, thereby facilitating the differentiation of pro-inflammatory Th17 cells[109]. This pro-inflammatory activity is further supported by the observation that PGE2 can also directly promote Th1 and Th17 differentiation and expansion after TCR triggering[110] by engaging EP2 and EP4 receptors, thus playing an important role in the pathogenesis of experimental autoimmune encephalomyelitis, and in a model of contact hypersensitivity[111]. The role of PGE2 in regulating immune responses is extremely complex and the net result of PGE2 activity depends on the specific PGE2 receptors expressed on the immune cells, their maturation state, PGE2 concentrations, the concomitant presence of other inflammatory factors, and the duration of the stimulus[97].

PGE2 is constitutively expressed in MSCs and its production is significantly induced after culture of MSCs with activated PBMCs or recombinant TNFα, IFNγ[40,84,112]. It has been demonstrated that PGE2 concentrations in MSC/PBMC co-cultures were associated with the anti-proliferative activity of MSCs[112,113]. Inhibition of PGE2 synthesis by the use of the COX2 inhibitors indomethacin or NS398 significantly abrogated the anti-proliferative activity of MSCs against both mitogen induced or antigen-specific T-cell proliferation[40,84,114]. Furthermore, genetically ablation of *COX2* in MSCs completely abrogate the capacity of MSCs to ameliorate neuroinflammation in a pre-clinical model of traumatic brain injury[113]. The production of PGE2 in MSCs has also been associated with the capacity of MSCs to induce macrophage polarization toward an M2 profile[115,116], to shift the polarization of T cells toward a Th2-type differentiation with inhibition of IFNγ and stimulation of IL-4 production[40], and to reduce TNFα production while increasing IL-10 secretion in LPS-stimulated DCs[40].

1.2.2.3 Tumour necrosis factor-stimulated gene 6 (TSG-6)

TSG-6 is a 35 kDa glycoprotein[117] which can be secreted in human fibroblasts, synoviocytes, chondrocytes, vascular smooth muscle cells or proximal tubular epithelial cells after induction by several stimuli including TNFα and IL-1, depending on the cell type considered[117–121]. Also PBMCs are able to produce TSG-6 in the presence of inflammation and LPS[117,118]. TSG-6 could exert an anti-inflammatory activity, as demonstrated in several *in vivo*

models of acute inflammation or antigen-induced arthritis[122–125]. Despite the variety of potential mechanisms employed by TSG-6 to deliver its anti-inflammatory activity[122,126–128], the specific pattern yet remains poorly elucidated. It is important to highlight that the effect seems to be mainly directed against the innate immunity and the acute phase of inflammation with a specific activity against neutrophil influx[122–124,126,129], without hampering the antigen-specific T-cell response[125,129,130]. However, an activity on the adaptive immune system cannot be completely ruled out, since a reduction of disease-specific antibodies in the serum of experimental animals with collagen-induced arthritis was also observed in the presence of TSG-6[124,129].

MSCs constitutively express TSG-6 at very low levels and this production is significantly increased in the presence of inflammatory cytokines such as TNFα[131–133] or bone morphogenetic protein 2[134]. TSG-6 production by MSCs was able to polarize macrophages toward an M2 phenotype[133], with inhibition of the secretion of TNFα *in vitro*[132,134] which was dependent on the reduction of the translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in macrophages mediated by the interaction between TSG-6 and CD44. The secretion of TSG-6 seems to be part of the immunosuppressive armamentarium of MSCs, at least in models where acute inflammation plays a central role. Indeed, it has been reported that MSCs were able to significantly attenuate the inflammatory infiltration in a mouse model of peritonitis or inflammatory bowel disease (IBD), and this property was completely abrogated when TSG-6 expression was genetically silenced in MSCs by the use of specific small interfering Ribonucleic acid (RNA)[132,133].

Notably, MSCs are induced to secrete large amount of TSG-6 after aggregation in microspheres upon activation of the Caspase1/NF-κB/IL-1 inflammasome signalling pathway[135,136]. This observation has important implications on understanding the complex mechanisms employed by MSCs to deliver immunosuppression after infusion, since MSC aggregation and TSG-6 production spontaneously occur *in vivo*[136], upon the formation of micro-emboli in the pulmonary vasculature after MSC intravenously injection[131]. Remarkably, MSCs trapped in lungs and activated to secret TSG-6 were able to reduce the infiltration of neutrophils in the myocardium and plasmin activity in the serum in a preclinical model of myocardial infarct[131]. This activity, which translated in significant reduction of infarct size and improvement of heart function, was completely abrogated when TSG-6 was silenced in the transplanted MSCs.

1.2.2.4 Transforming Growth Factor-β (TGF-β)

TGF-β is a pleiotropic factor consisting of three different isoforms (TGF-β1-3) with effects on different biological processes including immunological responses[137]. In the canonical TGF-β signalling, ligation of TGF-β to type II serine/threonine kinase receptor initiates a cascade of signals leading to activation of three cytoplasmic Smad proteins (Smad 2, 3 and 4) [138]. Alternatively, non-canonical TGF-β signalling cascades seem to mediate cancer progression when the canonical pathway is disrupted[139]. Since the first observation that TGF-β could inhibit IL-2 stimulated expansion of T

lymphocytes[140], strong evidence proved the pivotal role of this cytokine in the regulation of the immune system.

T cell proliferation of naïve T cells is strongly inhibited by TGF-β, mainly through blockage of IL-2 production[140]. Nevertheless, since very high doses of IL-2 to T cell culture only partially abrogated the anti-proliferative activity of TGF-β[140], also other pathways, involving the cyclin-dependent kinase inhibitors *p21* and *p27*, have been described[141]. Activated T cells are minimally sensitive to the antiproliferative activity of TGF-β which is however restored in the concomitant presence of IL-10[142].

TGF-β is able to hamper the differentiation of $CD4^+$ T cells toward both a Th1[143] or Th2[144] phenotype, and can affect the ability of $CD8^+$ T cells to mature into functional cytotoxic cells by inhibition of perforin[145] and FAS ligand[146] expression. This activity on T cell differentiation was confirmed *in vivo* by the observation that mice bearing TGF-β1 Receptor $II^{-/-}$ T cells developed a systemic autoimmune disease associated with an increased number of fully differentiated Th1 and Th2 T cells and spontaneously activated $CD8^+$ T cells able to produce effector cytokines[147].

TGF-β also plays an important role in the regulation of Treg cells. It has been shown that TGF-β1 can induce *in vitro* Treg conversion from naïve T cells via induction and maintenance of *Foxp3* expression[148,149]. Furthermore, TGF-β seems to be important in the maintenance of natural Treg cells (nTreg), since $TGF\text{-}\beta^{-/-}$ mice present reduced number of peripheral Treg cells[150]. This activity

seems to be dependent by a stabilization of Foxp3 expression in Treg cells mediated by the canonical TGF-β signalling pathway[151].

Although being mainly a suppressor factor on T cell functions, TGF-β has also an important role in the initiation of differentiation and Th17 cells[152] which secrete an array of cytokines involved in inflammation and autoimmunity[153,154].

As already mentioned, TGF-β has pleiotropic effects which influence not only T cells but also other components of the immune system, thus greatly extending the relevance of this cytokine in the fine tuning of immunological responses. Some of these effects will be summarized in the next part of the paragraph.

NK cells are strongly inhibited by TGF-β via attenuation of their IFNγ production and cytolytic activity[155,156].

The presence of TGF-β during DC maturation leads to accumulation of DCs with reduced antigen-presenting functions due to the retention of an immature phenotype characterized by low levels of CD1c and co-stimulatory molecules CD80 and CD86[157,158].

As for T and DCs cells, the activity of TGF-β on macrophages depends on their maturation state. Generally, during the acute phase of inflammation

TGF-β acts as a recruiting factor for monocytes[159,160] via induction of adhesion molecules on monocytes and facilitation of monocyte transmigration to the site of injury[161], whereby they are activated to produce inflammatory cytokines[159,162]. As the inflammatory response mounts and monocyte/macrophages mature, TGF-β suppressive activity starts to prevail. This inhibition affects macrophages at several levels of their activities such as phagocytosis[163–165], LPS-induced cytokine secretion[166], reactive oxygen[167] and nitrogen[168] species production, and antigen presenting functions[169,170].

The complexity of the activities mediated by TGF-β is further increased by the fact that most of the cells susceptible of its effects can secrete it in the microenvironment. Furthermore, also non-classical immunologic cells can produce these cytokines.

MSCs constitutively produce TGF-β in specific culture conditions[171] and its secretion has been described as an important mechanism employed by MSCs to mediate immunomodulatory effects. As already mentioned in the paragraph of the MSC immunobiology, MSCs exert potent anti-proliferative activity against T cells *in vitro*. This activity is mainly mediated by soluble factors[41], however it has been shown this inhibition to be significantly stronger in the presence of MSCs/T cell contact[172]. Notably, TGF-β plays an important role in both mechanisms. Indeed, T cell proliferation is partially restored in a transwell system in the presence of blocking antibodies against TGF-β1[41], but

also seems to be responsible of the additive anti-proliferative activity observed when MSCs and T cells are in contact[172].

As for other soluble factors employed by MSCs, also TGF-β is implicated in the MSC activity of re-shaping the microenvironment into an immunosuppressive niche via interaction with other cells of immunological significance. In a mouse model of ovalbumin (OVA)-induced Th2-type lung inflammation, MSCs were able to reduce the principal hallmarks of the disease (airway hyperresponsiveness and eosinophilic accumulation in bronchoalveolar lavage [BAL]). This effect was dependent on the polarization of alveolar macrophages toward an M2 phenotype which was mediated by the production of TGF-β by MSCs[173]. Moreover, several studies have described the role of the TGF-β secreted by MSCs in the induction and expansion of Treg cells[174–176].

1.2.2.5 Role of other immunomodulatory factors

In association to the factors described above, other molecules have been described as mediators of direct MSC immunosuppressive activity.

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme controlling the degradation of heme into biliverdin, free iron and carbon monoxide. HO-1 can exert potent anti-inflammatory effects by inhibiting the production of TNFα, IL-1β, and macrophage inflammatory protein-1β while inducing secretion of IL-10 in LPS-stimulated macrophages both *in vitro* and *in vivo* through the

mitogen-activated protein kinase pathway[177]. Furthermore, HO-1 hampers DCs maturation and their capacity to stimulate T cell proliferation in response to alloantigens[178], and its overexpression in rats receiving heart transplant leads to long-term allograft survival[179]. Importantly, HO-1 has been demonstrated to exert pro-tumour effects, by inhibiting $CD8^+$ cell responses against cancer cells[180], thus its inhibition is now considered an important target of cancer immune check points[181]. HO-1 plays an important role in MSC mediated immunosuppression, as demonstrated by the observation that its inhibition could restore proliferation of human PBMCs when stimulated in Mixed Lymphocyte Reactions (MLR) in the presence of MSCs[182].

MSCs can secrete the soluble isoform 5 of the non-classic HLA class I molecule HLA-G (HLA-G5). HLA-G plays an important role in the maintenance of the maternal tolerance toward the foetus[183], and its expression after solid organ transplantations is fundamental in the acceptance of the graft[184,185]. It has been demonstrated that HLA-G can have several immunosuppressive activities, including hampering the cytotoxic activity of $CD8^+$ cells or NK cells[186], or impairment of DC maturation[187]. MSC production of HLA-G5 requires IL-10 and cell-contact between MSCs and activated T cells, and it inhibits T cell proliferation and impairs NK cells cytotoxic activity and IFNγ production[188].

Other molecules with immune suppressive activities described as produced by MSCs are galectin 1[189], 3[190] and 9[191], and they could play a role in reducing both acute and chronic inflammatory responses.

The direct suppressive activity of MSCs is not limited to the secretion of soluble factors. Indeed, MSCs can express ligands on their surface which interact with their counterpart on the membrane of immune cells, thus directly transmitting inhibitory signals. Examples of such mechanisms are the expression of programmed death-ligand 1 (PD-L1/B7-H1/CD274) and the membrane-bound HLA-G1. The expression of the former can be induced by IFNγ in MSCs and the interaction between PD-L1 with its receptor PD1 on T cells mediates inhibition of both proliferation and effector functions of activated T cells[85]. The latter is expressed by both BM-MSCs and MSCs obtained by foetal liver and can induce arrest of the cell cycle in T cells and inhibit their IFNγ production[192].

1.2.2.6 Interplay between factors

In the previous paragraphs different molecular patterns have been extensively described. An important aspect to underline is that all these factors are part of a well-known system of the “innate tolerance” which can play an important role in different conditions, ranging from autoimmunity to cancer immune escape. The fact that most of them are employed by MSCs,

makes it conceivable that MSCs are important components of this tolerogenic systems.

It is unlikely that any of these factors alone may represent the necessary and sufficient candidate accounting for the whole immunomodulating activity mediated by MSCs, especially in complex scenarios like *in vivo* settings. It is more likely that each factor concurs synergistically to the final production of an immunosuppressive niche. Same conditions can also activate different patterns, thus highlighting the remarkable complexity and redundancy of the molecular pathways activated in MSCs. Same stimuli can elicit different factors. For example, TNFα and IFNγ can induce IDO[61–63], PGE2[40,84,112], TSG-6[131–133]. Similarly, formation of MSC micro-spheres can act as driver for the induction of both PGE2 and TSG-6[116,136]. Furthermore, IDO, PGE2, TSG-6 and TGF-β exert specific activities on third cells (macrophages and Treg cells for instance) which in turn can produce any of these factors. It is then conceivable that the respective importance of these molecular patterns may have different levels of relevance, depending on the specific scenario or inflammatory context where MSCs are used.

1.2.3 MSC immunosuppression by indirect activity on regulatory cells

As emerged from the previous paragraph, MSCs can produce a wide range of factors which not only have direct immunomodulating effects but can also

play a role in the interaction between MSCs and other immune cells such as macrophages, DCs and Treg cells through a mechanism which can be defined as the “indirect immunosuppressive” activity of MSCs. The education of these cells orchestrated by MSCs will gather a network of regulatory immune cells which will support and maintain for longer terms the immunosuppressive niche created by MSCs through soluble factors.

1.2.3.1 Expansion of T cells with regulatory properties

The concept of a subpopulation of T-cells with regulatory properties was first presented in the 1970s[193]. Since then, the pivotal role played by these cells in immune homeostasis and maintenance of self-tolerance has been extensively confirmed both in mice and humans; and the term Treg cells was then introduced[194,195]. Initially, Treg cells were thought to be a distinct subpopulation within $CD4^+$ T cells specifically expressing both the IL-2 receptor α-chain $CD25^+$[196,197] and FOXP3[198–200] in opposition to conventional effector $CD4^+$ T cells. However, it is now clear that the expression of both markers is not uniformly associated with suppressive functions in $CD4^+$ T cells[201–203].

Treg cells consist of two different subtypes of cells described *in vivo*: thymus-derived or nTreg and adaptive or induced Treg cells (iTreg) generated in periphery[194,204].

Both classes of Treg cells can inhibit any step to the acquisition of effector functions of virtually any cell of the immune system. Their mechanisms of activity are the object of intensive research and seem to include cell contact direct cytotoxicity, deprivation of essential amino acids or growth factors from the environment, and secretion of soluble factors with inhibitory properties[194,205]. nTreg and iTreg cells seem to have similar but not identical transcriptomes[206,207], and this may reflect functional disparities and non-redundant roles[207,208]. Treg functions are associated with a peculiar epigenetic status of the Treg-specific demethylation region partially dependent by TGF-β[209–211]. This CpG hypomethylation pattern regulates the stability of FOXP3 expression and other specific Treg signatures and they are partly shared by nTreg and *in vivo* iTreg cells, but they are not detected in *in vitro* generated iTreg[212]. These observations highlight the close relationship between nTreg and *in vivo* generated iTreg, which are thought to cooperate to maintain immune homeostasis.

nTreg cells develop in the thymus after interaction between MHC class II molecules and cognate TCR with high avidity[213–215]. They seem to have a crucial role in the maintenance of peripheral self-tolerance, as demonstrated by the observation that thymectomy in neonatal[216] or adult[217] normal animals results in the development of autoimmunity which can be reverted by the infusion of syngeneic $CD25^+$ T cells[216]. Definitive evidence that these cells originate in the thymus and then persist in periphery has been provided by the finding that infusion of thymocytes depleted of $CD4^+CD25^+$ cells into athymic nude mice leads to several autoimmune diseases[218].

In vivo generated iTreg cells are otherwise conventional $CD4^+$ T cells which acquire FOXP3 expression and tolerogenic phenotype in periphery.

Regardless of the specific condition considered, several different molecules can play a role for the generation of iTreg *in vivo*, including TGF-β, IL-2 or retinoic acid[204,219]. Similarly, also nTreg expansion can be promoted by different factors[194]. Among these factors, IDO can have important links with nTreg and iTreg cells biology. It has been demonstrated that DCs expressing IDO can mediate iTreg cell generation *in vitro*[80,220], maintain their suppressive phenotype with inhibition of re-programming toward inflammatory functions both *in vitro* and *in vivo*[221], and activate potent immunosuppressive properties in pre-existing, resting nTreg cells[222].

One of the properties of MSCs is the ability to generate a niche able to favour the expansion of Treg cells or their generation *de novo*, both in a direct or an indirect manner. Indeed, with the flourish of studies focusing on MSC immunosuppressive mechanism of action, it became apparent that part of the MSC activity can be associated with an increment of T cells with regulatory properties. Early studies demonstrated that MSCs in co-culture with both autologous or third-party PBMCs induced expansion of a population of $CD4^+$ T cells with suppressing properties and co-expressing CD25 and CTLA-4[223] or CD25 and glucocorticosteroid-induced TNF receptor family member[40], two markers upregulated in Treg cells[224] and whose expression seems to be under the control of FOXP3[225,226]. These findings were confirmed in other studies where *FOXP3* expression was detected in expanded $CD4^+CD25^+$ T

cells[227,228]. But the important role played by the MSC/Treg interaction has been comprehensively supported by the plethora of *in vivo* studies demonstrating a tight relationship between MSC therapeutic activity and Treg expansion. The robustness of this observation comes also from the fact that similar findings were reported by using different species and disease models. Indeed, Treg expansion following MSc treatment has been described in several pre-clinical models of autoimmune diseases[174,229,238,239,230–237].

In most of these studies the Treg importance in the MSC-mediated immunosuppression was evaluated indirectly, i.e. the loss of MSC efficacy was obtained by blocking those factors leading to Treg increase. Furthermore, the depletion of Treg cells from MSC recipient completely impaired the MSC therapeutic activity[229,236].

Importantly, it has to be noted that similar increase of the Treg cell population has been reported also in human patients after MSC administration. In their safety and feasibility pilot study on the use of autologous MSCs in patients receiving kidney allografts from living donors, Perico and colleagues[240,241] treated four patients and found that graft functions remained stable after transplant and MSC treatment, supporting the safety of the procedure. Importantly, all patients showed a progressive increase of the Treg population[240,241]. Similar findings were reported by Zhao et al. in a study where 28 patients affected by steroid resistant GvHD were treated with MSCs. Median MSC dose was $1x10^6$/Kg body weight and the cells were infused weekly until complete response or when a maximum of 8 doses were

reached. Overall response rate was 75% and was significantly higher than what observed in a group of 19 similar patients but not treated with MSCs. Importantly, the monitoring of the immune cells in peripheral blood showed an increase of the level of Treg cells after MSC treatment in comparison with pre-treatment values in the same patients or with not-treated patients[242]. No analysis on the possible association between levels of Treg and response to MSCs was reported.

Whether the increase of the Treg cells induced by MSCs is the result of the generation of new iTreg cells or conversely of the expansion of pre-existing nTreg is largely unknown. This is not a semantic problem. Indeed, a better understanding of the characteristic of these Treg cells would be important from a translational perspective and for understanding the mechanisms leading to durable clinical responses after MSC treatment. Indeed, the stability of iTreg tolerogenic phenotype can be highly variable and can be re-programmed toward pro-inflammatory phenotypes[194]. Only few *in vitro* studies tried to address this question, and the observation that $CD4^+FOXP3^+$ could be obtained from $CD4^+CD25^-$ cells suggest that, at least in specific culture conditions, MSCs can generate iTreg cells[243,244]. The very recent finding that Treg cells obtained from $CD4^+CD25^-$ cells exhibit enhanced demethylation of the Treg-specific demethylation region[245] seems to confirm the similarity of these MSC-generated iTreg cells with those described *in vivo* and characterized by stable and potent suppressive activity[204].

Another issue still subject of intense research is how the interaction between MSCs and T cells leads to the increase of Treg. While a few studies pointed out the requirement of cell contact[175,188,243], in other studies this seems to be dispensable[245,246]. Furthermore, other findings suggested an indispensable and non-redundant role played by soluble factors such as TGF-β *in vitro*[176,230,232,235] or IDO *in vivo*[238], since the MSC-mediated increase of Treg cells was completely abrogated by means of TGF-β blockage using neutralizing antibodies[230,232,235] and gene knock down[176], or either IDO chemical inhibition or knock out engineering[238], respectively. However, these latter studies did not directly address the necessity of cell-contact, thus leaving unanswered the question on the actual importance of this factor.

MSCs can indirectly induce Treg cell expansion through interaction with other cells. An important aspect that should be considered is also the concomitant presence of other immune cells which can participate in the process of Treg expansion after being in contact or in close vicinity with MSCs. Indeed, in their study of the MSC-T cell interaction, English and colleagues demonstrated that Treg expansion could be obtained only by cell-cell contact via production of PGE2 and TGF-β, however the cell contact was dispensable when MSCs were mixed with an unfractionated population of PBMCs[175]. This apparent discrepancy could be explained by the role played by other mononuclear cells which, under instruction from MSCs, become mediators of the Treg expansion. Similar findings were reported by Patel et al., whereby the induction of Treg increase by MSCs was dependent on TGF-β produced by MSCs upon contact with breast cancer cells[176]. Furthermore, indeed, it has

been extensively shown that MSCs can modulate the activity of APCs (including macrophages[83,116] or DCs[244,247]) leading them toward a regulatory phenotype which in turn mediate the development of Treg cells.

MSCs are also capable to induce *in vitro* expansion of other T cells with regulatory properties, such as IL-10-secreting T regulatory type 1 (Tr1) and TGF-β-secreting T helper 3 cells (Th3) through the production of HO-1[248].

All these studies clearly demonstrate that Treg expansion is an important part of MSC immunosuppressive armamentarium. Several mechanisms can lead to Treg increase, much has been unveiled but more work is still needed for a comprehensive understanding of the relative importance of each factor in different inflammatory microenvironments or clinical settings.

1.2.3.2 Education of the Mononuclear Phagocyte System (MPS)

As seen in the previous paragraph, MSCs can orchestrate the expansion of a regulatory population of T cells, partly secreting those same factors which have potent suppressive activity also in a direct manner. Similarly, MSCs can also guide the generation of tolerogenic myeloid derived cells. These cells are grouped in what is defined the MPS. Historically, members of this system are DCs, monocyte-derived cells and tissue-resident macrophages[249]. When the MPS was conceived in early 70s[250] and after the discovery of DCs[251] and

their inclusion in this system[252], these three types of cells were grouped together because of their phagocytic capacities and because it was thought that all originated by a common precursor, namely the circulating monocytes. In fact, it is now appreciated that monocyte-derived cells, macrophages and DCs belong to three different families of cells with different precursors. However, all share many overlapped functions[249].

Peculiarity of the cells of the MPS system is their extremely high plasticity and capacity to respond to environmental signals with a broad repertoire of effector functions. Indeed, shaped by surrounding cues, MPS cells can change their phenotypes and orchestrate the quantitative and qualitative type of immune response toward immunity or tolerogenesis induction. MSCs are able to interact with all cells of the MPS system and instruct them to acquire potent anti-inflammatory and immune suppressive phenotypes.

1.2.3.3 MSC interaction with monocyte-derived cells and tissue-resident macrophages

Tissue resident macrophages and monocyte-derived cells are very close relatives. At least in a steady-state condition, tissue resident macrophages originate from precursors which migrate to peripheral tissues during embryonic development[253–256], and are maintained through self-renewal throughout life[257].

Monocyte-derived cells are a heterogenous population of cells traditionally described as classical or non-classical monocytes. It has been demonstrated that classical monocytes derive from a common precursor[258], whereas non-classical monocytes seem to mainly patrol the vasculature bed and their ontogeny is still debated.

Both populations of cells are highly plastic, and the essence of this complexity is expressed by the concept of macrophage polarization into M1 (or classical activation) and M2 (alternative activation), notion used to describe extreme phenotypic states resulting from a continuum of stimuli[259]. M1 macrophages were initially described as those induced in the presence of IFNγ combined with LPS or TNFα and mediated Th1 type responses with production of high levels of IL-1β, IL-12, reactive nitrogen intermediates, or reactive oxygen species. Conversely, M2 macrophages were induced in the presence of IL-4 and IL-13, immunocomplexes, or IL-10 and produced IL-10, TGF-β, and polyamines, mainly mediating immune regulation, extracellular matrix remodelling and tissue repair, and Th2 responses[259,260]. It is now emerging as a far more complex process and this concept has been challenged and integrated with novel insights based on more advances in transcriptomics, proteomics and the use of genetically modified mouse strains which demonstrated the presence of entities with intermediate functions[261]. However, this dichotomy is very useful because it underlines the functional differences which monocyte-derived cells and macrophages can show depending on the specific microenvironment where they are activated. Thus, this terminology will be used in the rest of this dissertation, whereby the most

important factors driving a tolerogenic phenotype (M2 polarization) will be presented.

MSCs can recruit monocytes at sites of injury and inflammation. In the presence of low doses of LPS or other TLR ligands in the bloodstream, MSCs are induced to express monocyte chemotactic protein-1 which is required for mobilization of monocytes from the BM in response to bacterial infections[262]. Once at the site of injury, factors produced by MSCs drove the differentiation toward an M2 phenotype of the recruited monocytes which eventually enhanced wound healing in a excisional wound healing model[263].

Several studies have confirmed the capacity of MSCs to promote M2 polarization *in vitro*. BM-MSCs in co-culture of blood-derived monocytes induced upregulation of IL-10 and CD206 and downregulation of IL-12 and TNFα, all markers of M2 phenotype[264]. Similar results were obtained also using MSCs from other sources such as umbilical cord[265] or gingiva[266], thus highlighting that this important property is shared by MSCs from different origins. Notably, MSC-primed monocytes/macrophages seemed to be important in the anti-proliferative effect of MSCs *in vitro*, since macrophage depletion in the MSC/PBMC co-culture resulted in restoration of PBMC proliferation[83,265]. The mechanisms employed by MSCs to mediated the M2 differentiation have been identified in the MSC production of IDO[83], PGE2[267], and IL-6[268]. However, more recently also a different mechanism has been proposed. In their study, Selleri and colleagues showed that MSCs were able to re-programme monocyte differentiation by secreting large amount of

lactate which eventually modified monocyte metabolism, thus leading to M2 polarization. Reduction of lactate production by MSCs, completely abrogated this effect[269].

The ability of MSCs to educate monocyte-derived cells and macrophages has been confirmed *in vivo*. In their experiments, Nemeth et al. demonstrated that MSC effectiveness in reducing mortality or organ failure in a mouse model of sepsis was dependent on the presence of macrophages in MSC-treated mice. After activation of TLR4, MSCs were induced to produce PGE2 which stimulated IL-10 production in MSC-recipient macrophages through the interaction of PGE2 with its receptors EP2 and EP4. Macrophage depletion, inhibition of IL-10 or use of $TLR4^{-/-}$ MSCs completely abrogated the therapeutic effect of MSCs[270].

We have already discussed the role of TSG-6 in the MSC property to deliver immunosuppression *in vivo*. This effect is mediated by the activity of this molecule on the macrophage populations of MSC-recipient. In a mouse model of zymosan-induced peritonitis, a study of the Prockop's group showed that MSC treatment was able to reduce macrophage infiltration in the peritoneum. This effect was mediated by MSC-derived TSG-6 which, upon interaction with CD44 on macrophage surface, hamper NF-κB translocation into the nucleus and transcription of inflammatory genes in macrophages[132]. The same group demonstrated the importance of these MSC-primed monocytes using a mouse model of corneal allotransplantation and experimental autoimmune uveitis. MSC treatment conferred protection

against both diseases and the beneficial effect was mediated by the increase of immune suppressive monocyte-derived cells induced by MSC-produced TSG-6. Notably, when MSC-conditioned monocytes were adoptively transferred into second animals, they showed therapeutic activity against both corneal rejection and uveitis[271].

1.2.3.4 MSC interaction with DCs

DCs represent a heterogenous population of antigen presenting cells which are central in the homeostasis of immune system. Indeed, while its regulation of the adaptive immune system is well known, only recently an important role also in the innate responses has been decsribed[272–274]. Traditionally, DCs have been categorized in two distinct subgroups: plasmacytoid DCs (pDCs)[275,276] and conventional or classical DCs (cDCs)[249].

As in monocyte/macrophage cells, contrasting functions (immunity versus tolerogenesis) can be demonstrated also in DCs. Indeed, in this subset of cells alteration of the process of maturation can lead to opposite outcomes. In the presence of inflammatory signals such as pathogen-associated molecular pattern molecules, damage-associated molecular pattern molecules or inflammatory cytokines, DC maturation is triggered, and these cells express high levels of MHC molecules in association of the co-stimulatory molecules CD80, CD86 and CD40, secrete large amount of IL-12, and mediate T cell activation[277,278]. Conversely, in the presence of

suppressive cues (or in the absence of inflammatory stimuli) DCs maintain an immature phenotype and become strongly immunosuppressive[275].

DCs can exert their immunosuppressive activity not only by directly mediating differentiation of hypo responsive T cells but also inducing expansion of Treg cell populations. Indeed, cDCs were important in the support of nTreg development in the thymus through inhibition of nTreg apoptosis[279], and pDCs from Peyer's patches stimulated with cytosine-phosphate-guanosine motifs were able to stimulate formation of iTreg from $CD4^{+}CD25^{-}$ T cells[280].

The role of IDO in DC functions seems to be complex with opposite activities. While the production of IDO by DCs was suggested to be essential for the complete maturation and acquisition of chemotactic antigen presenting capacity in the presence of inflammatory stimuli[281], IDO produced by DCs has been implicated in the generation of iTreg and tolerance induction[282]. Spleen tolerogenic cDCs have be shown to be responsible of the expansion of iTreg cells *in vivo* through the production of IDO, and these iTreg cells mediated the induction of oral tolerance to the fed antigen[283]. Importantly, Treg cells can also stimulate IDO production in DCs[64,65], thus further contributing to the generation of an immunosuppressive milieu.

The creation of such a microenvironment is the hallmark of MSC activity, as already described in previous paragraphs. It is then obvious that this MSC

capacity has also significant impact on DC ability to mature and to acquire a tolerogenic phenotype.

MSCs can impair the differentiation of cDCs from $CD34^+$ cord blood cells[284] or from peripheral blood monocytes in the concomitant presence of granulocyte-macrophage colony-stimulating factor plus IL-4 (conditions usually used to differentiate DCs from monocytes)[285]. DC maturation is completely impaired in the presence of MSCs with significantly reduction of expression of MHC proteins and co-stimulatory molecules[285–288]. Consistently, DC capacity to stimulate naïve T cell proliferation was weakened[286–288]. Notably, also in the presence of some features of maturation, such as a pro-immune IL-12/IL-10 secretion profile, DCs cultured in the presence of LPS and MSCs retained their phagocytic capacity and lost their capacity of form functional immunologic synapsis with T cells and stimulate activation of allogenic responses[289].

Several mechanisms have been described to explain how MSCs can mediate these effects with contrasting results. While a few studies found a dispensable role of cell-cell contact and pointed to IL-6 production as important mediator of MSC capacity to induce tolerogenic DCs[286,287,290], others demonstrated how inhibition of other factors, such as TSG-6[291] or PGE2[292], could abolish this effect. Notably, a partial role of cell contact interactions has also been reported. Indeed, generation of regulatory DCs by MSCs was partly mediated by activation of Notch signalling pathway in a contact dependent manner[288]. Accordingly, Jagged-1 and Jagged-2[293] have been described as playing a

role in the MSC/DCs interaction[294,295]. These discrepancies could be ascribed to the different experimental systems studied but also underline the possible redundancy of the different mechanisms.

Several studies confirmed the crucial role of tolerogenic DCs in mediating the immunosuppression delivered by MSCs *in vivo*. After infusion, MSCs hampered the DC homing capacity to lymph nodes by reducing the expression of the CCR7 and $CD49_d\beta1$, two molecules important for DC trafficking. Importantly, these DCs were unable to prime $CD4^+$ T cells *in vitro* and *in vivo*, and to cross present antigens to $CD8^+$ T cells *in vitro*[296]. Infusion of MSCs in combination with low doses of rapamycin significantly prolong allogenic heart and skin graft survival. This effect was associated with increase of Treg cells and tolerogenic DCs in spleens of treated animals[236].

Similar data were also observed in humans. A significant increase of cDCs was observed in GvHD patients 2 weeks after MSC infusion which remained stable at later time points. A more pronounced increase of immature DCs was observed in those patients responding to MSCs when compared with non-responding patients[297].

1.2.4 MSCs as tolerogenic and therapeutic agents

The characteristics described in the previous paragraphs ignited the interest in MSCs, considered as promising tools to control aberrant inflammatory

responses by producing an immunomodulating environment. As shown in Figure 1.2, consultation of the public registry of clinical trials at the U.S. National Institute of Health database (*at ClinicalTrials.gov*) shows a continuous increase of the number of new studies involving MSCs for the treatment/prophylaxes of immune mediated diseases which were registered between 2004 and 2010, with at least 10 new registrations thereafter.

The focus of this intensive research has been mainly centred around two aspects: 1) the use of MSCs to exert peripheral tolerance in contexts whereby this tolerance was altered *after* the use of MSCs (i.e. usefulness of MSCs as prophylaxes), and 2) the use of MSCs when an inflammatory or autoimmune response was already established *before* MSC infusion (i.e. MSC use as proper therapy to restore peripheral tolerance).

Figure 1.2. MSCs as therapeutic agents in immune-mediated diseases.

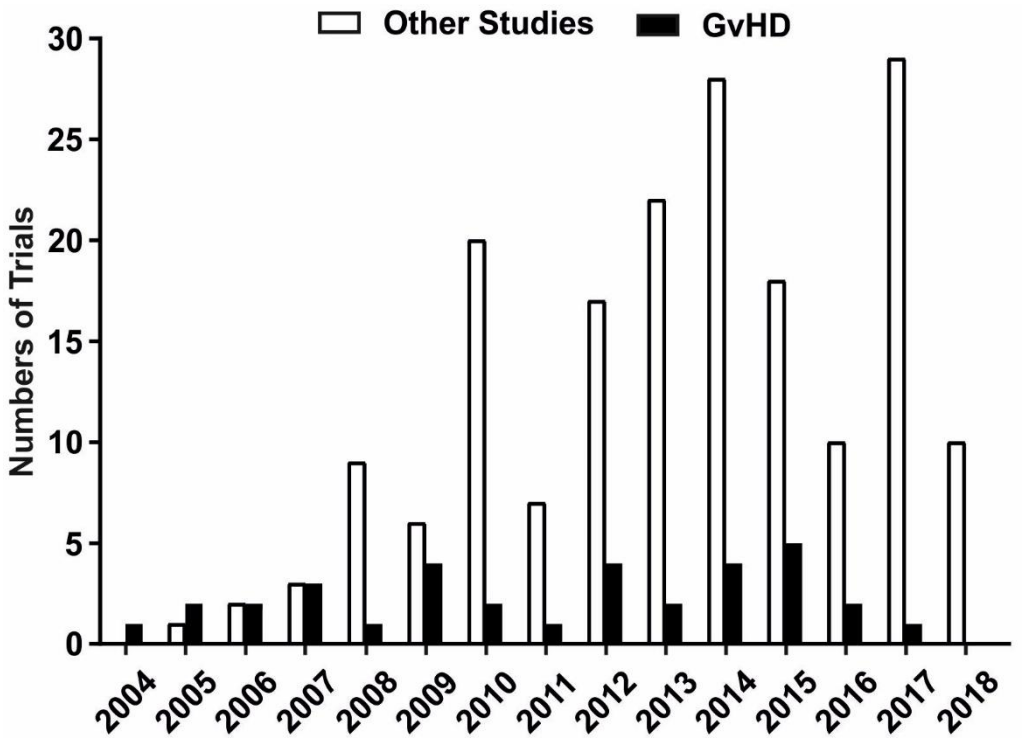

Figure 1.2. MSCs as therapeutic agents in immune-mediated diseases.

Number of Clinical trials registered at the U.S. NIH database registry (*ClinicalTrials.gov*) plotted according to the year of registration. Search was performed in February 2018 and included all studies whereby MSCs (Mesenchymal Stromal/Stem Cells) were used as drug for the treatment of GvHD or other immune-mediated diseases. Other diseases included: inflammatory lung diseases (including asthma and Chronic Obstructive Pulmonary Disease), Chron's Disease, Ulcerative Colitis, Systemic Sclerosis, Rheumatoid Arthritis, Rheumatic arthritis, Diabetes Mellitus, Cystic Fibrosis, Multiple Sclerosis, solid allograft rejection, Retinitis Pigmentosa, Sjogren

Syndrome, Systemic lupus erythematosus, neuromyelitis, engraftment of HSCT.

1.2.5 MSCs and its use as second line GvHD therapy

GvHD is a life-threatening complication of allogeneic HSCT, and currently represents one of the major factors limiting the success of this potentially curative option for haematological malignancies[298,299]. GvHD develops when donor alloreactive T cells respond to host antigens[300]. The most important factor determining GvHD frequency is the disparity of HLAs repertoire between donor and recipient[301]. However, also patients receiving fully HLA matched donor cells can develop GvHD. This depends on the recognition of minor histocompatibility antigens[302], allelic or sex-linked polymorphisms that act as transplantation antigens. Furthermore, genetic polymorphisms of genes encoding cytokines[303,304] or proteins involved in innate immune responses[305] can also contribute to increase the incidence of GvHD.

GvHD has been classified into acute (aGvHD) and chronic (cGvHD). Traditionally, aGvHD was defined as arising by day 100 after HSCT, while all forms developing after that cut-off were considered as cGvHD[298]. However, clinical manifestations of aGvHD could persist or develop later than the first three months and symptoms of aGvHD could arise in patients with pre-existing cGvHD. To address these issues, the National Institutes of Health Consensus Conference on GvHD proposed[306], and subsequently refined[307], a set of standardized criteria for the diagnosis and scoring of the severity of GvHD based on the assumption that only clinical features should define whether the clinical syndrome of GVHD is considered acute or chronic.

aGvHD consists in: 1) classic aGvHD, occurring within the first 100 days after HSCT; and 2) persistent, recurrent or late-onset aGvHD, incorporating those aGvHD forms arising after that time cut-off.

Organs typically involved in aGvHD are skin, liver and gut. However, a serious clinical syndrome resulting from diffuse and non-infectious lung injury has been described early after HSCT and is not recognised as manifestation of cGvHD[308–310]. This complication, termed idiopathic pneumonia syndrome, responds poorly to conventional treatments. Although histologic criteria of GvHD diagnosis are difficult to be met in lungs, there is accumulating evidence which supports the notion that also lung should be included among the target organs of aGvHD[311,312].

aGvHD complicates HSTC in 35-80% of transplanted patients, depending on the HLA mismatch between recipient and donor, source of haematopoietic stem cells, transplant conditioning regimen, and GvHD prophylaxis[298]. The severity of aGvHD is determined by using a scale of 4 grades based on the involvement and extent of the three main target organs: I (mild), II (moderate), III (severe) and IV (very severe). Grade III and IV aGvHD are those with the poorest prognosis with median overall survival between 25% and 5% at 5 years from GvHD diagnosis, respectively[298]. Apart from grade I skin aGvHD which is treated with topic steroids, treatment of choice of aGvHD is the use of systemic steroids at high doses with 60-80% of the patients responding to treatment, depending on the severity of the disease at diagnosis[313].

cGvHD is the most important long-term complication after allogenic HSCT, significantly affecting the quality of life of patients and with negative impact on their survival[314]. Its frequency varies between 30% to 70% of transplanted patients, depending on several factors including prior aGvHD diagnosis, type of transplant and source of haematopoietic cells[315]. cGvHD is divided in: 1) classic cGvHD, developed after 100 days and without concomitant symptoms of aGvHD; and 2) overlap syndrome, which includes all patients with diagnosis of cGvHD presenting concurrent manifestations of aGvHD (independently of the temporal relation with cGvHD)[307].

cGVHD is a syndrome characterised by variable manifestations which recall other known autoimmune disorders, such as bronchiolitis obliterans, chronic immunodeficiency, scleroderma, primary biliary cirrhosis, Sjögren's syndrome, or immune cytopenias[306,307].

Albeit the pathophysiology of cGvHD remains poorly defined, recent findings suggest a crucial role of naïve T cells dysregulation which leads to thymus damage and alteration of the antigen-presenting processes in the periphery. These initial factors would lead to the generation of aberrant T and B cell responses, production of auto and alloantibodies, and the activation of macrophages with deposition of extracellular matrix and development of fibrosis[316]. Manifestations of cGVHD are broadly variable and may be localized to a single site or be widespread, they can be self-limited and/or disappear without treatment[298,306,307].

1.2.5.1 The role of MSCs for the treatment of aGvHD

Currently, there is no standardized treatment for patients who do not respond to steroids and their prognosis is still very poor, with overall survival inferior to 20% at 2 years[317].

Thanks to their potent immunosuppressive activity *in vitro*, and after the report that a patient with grade IV GvHD resistant to several lines of treatment was able to achieve a dramatic improvement after multiple infusions of third-party MSCs in 2004[26], the interest in using MSCs in GvHD patients has sparked remarkably. Since then, at least one new clinical trial involving the use of MSCs to mitigate GvHD has been registered every year at ClinicalTrial.gov with a peak of 5 different studies started in 2015 (Figure 1.2). To date (last analysis in October 2018), a systematic search of the literature which includes both retrospective and interventional studies on the use of MSCs to treat aGvHD has identified 24 different studies with at least 10 patients enrolled (Table 1.1).

When also small case reports[318–326], a total of 835 aGvHD patients have been reported as treated with MSCs and more than 1000 MSC doses administered. Both paediatric and adult patients were treated with age ranging from 2 months to 72 years.

All aGvHD patients were steroid resistant. Only one study used MSCs as first line treatment in association with steroids[327].

MSCs could successfully be expanded from disparate tissues, spanning from BM[242,297,329–338,319,339–345,320–322,325–328], umbilical cord (UC)[318,346], adipose tissue (AT)[323,324], or placenta[347]. While BM has been the first MSC source ever described and the most employed thus far, this data is remarkable since umbilical cord and adipose tissue may be considered more “affordable” alternative sources in terms of manufacturing logistics and costs in comparison with BM. Indeed, obtaining MSCs from UC or AT tissues has important advantages. First, the invasive BM harvest procedure, associated with (minimal) risk for donors, can be spared. Secondly, both UC-MSCs and AT-MSCs can be obtained from tissues which are currently otherwise discarded, and, at least for UC, also from samples previously frozen before isolation[348,349]. Third, they have higher proliferative capacity and longer life-span *in vitro* with higher cells yielded per expansion[37,350]. As already described in the paragraph describing the MSC immunobiology, the origin of the MSCs does not seem to affect their anti-proliferative and immunological properties *in vitro*. The data reported in these studies, despite the small number of patients treated with UC[318,346], AT[323,324] or placenta[347], have shown similar response rates to those reported in similar studies where BM-MSCs were used (Table 1), thus supporting the role of these sources as valid alternatives for clinical-grade MSC production.

MSC use in aGvHD seems to be safe, since no acute adverse reactions related to MSC infusion have been reported, regardless the source of the MSC used, the treatment schedule or MSC dose. These findings further confirmed a previous comprehensive meta-analysis performed on 8 different

controlled clinical trials, whereby MSCs were used for the treatment of several diseases (including GvHD, stroke, cardiomyopathy, solid organ transplant and healthy volunteers). The studies reported were performed in 14 different countries in Asia, the Middle East, Europe, and North America. The meta-analysis did not find any association between MSC administration and acute infusional reactions, organ complication, infections or death[351]. The potent immunosuppressive properties of MSCs raise serious concerns on the residual ability of the patient to respond to infections and the underlying malignancy. In their study, Zhao and collaborators prospectively compared the frequency of infectious episodes (including Epstein–Barr virus and Cytomegalovirus reactivations) and cancer relapses in MSC-treated and non-treated patients with a follow-up of one year and did not find any difference between the two groups[242]. Another group found that both the incidence of virus reactivations and latency from the start of steroid treatment were similar in patients treated with MSCs and historical controls[332]. Interestingly, the competing risk analysis to test the likelihood of infection or death during the first 100 days after MSC infusion found no difference between MSC responders and non-responders[297]. These findings are corroborated by the available published data which support the notion that MSCs do not lead to an increase in both infection severity or frequency, or number of relapses in MSC treated patients[337,338,341], when compared with similar cohorts of patients but treated with different treatments[352].

Outcomes of patients treated with MSCs in the reported studies are summarized in Table 1. It is not possible to directly compare these studies

due to the heterogeneity of the patients enrolled. Furthermore, while most of the studies were prospective[242,297,336–342,321,322,327,329,331–334], some of them were retrospective[325,328,330,335,343–345,347] or case reports[318,319,323,324,326,353]. However, data on MSC efficacy have been extremely encouraging with overall response rates between 33% and 100% (median 72%). Broad variations were reported regarding patients' overall survival (OS). Although a few studies showed an OS at 2 years after first MSC infusion between 17% and 35%[329,338,339,343,345], most of them reported an increase of the survival of patients after treatment with MSCs, with OS rates ranging from 40% up to 76%[242,297,337,340,341,320,327–331,334,336].

Despite the broad variability in the terms of outcomes, there is a remarkable homogeneity on the results in every study when the OS is considered in the sub-categories of responding and non-responding patients. Indeed, patients who responded to MSCs consistently exhibited a significantly longer OS, with survival rates at 2 years higher than 50%[297,320,327–331,339].

	Patients		MSC			Outcome		
Publication	**N**	**Median age (range)**	**Source/ n of donors**	**Dose (x10^6/Kg)**	**Number of Infusions Median (range)**	**CR (%)**	**PR (%)**	**NR (%)**
Prasad, 2011[328]	12	5 (0,4-15)	BM/4	2.00-8.00	8 (2-21)	17	50	33
LeBlanc, 2008[329]	55	22 (0.5-64)	BM/45	0.40-9.00	2 (1-5)	54	16	29
Ball, 2013[330]	37	7 (0.7-18)	BM/NRe	0.90-3.00	2 (1-13)	65	22	13
Kurtzberg, 2014[331]	75	8 (0.2-17)	BM/7	2.00	NRe (8-12)	NRe	NRe	NRe
TeBoom, 2015[297]	48	44.9 (1.3-68.9)	BM/multiple	1.80 (0.90-2.50)	3 (1-4)	25	50	25
Chen, 2012[346]	19	NM	UC/NRe	0.60-7.00	NRe	58	21	21
Kebriaei, 2009[327]	31	52 (34-67)	BM/multiple	2.00-8.00	2 (2)	77	16	7
Erbey, 2016[335]	33	7 (3-18)	BM/68	0.50–2.80	2 (1-4)	54	21	25
Muroi, 2013[336]	14	52 (4-62)	BM/multiple	2.00	8 (8-12)	57	36	7
Muroi, 2016[337]	25	35 (5-66)	BM/multiple	2.00	8 (4-16)	24	36	40
Sanchez-Guijo, 2014[342]	25	NM (20-65)	BM/24	1.10 (0.70-1.31)	4 (2-4)	44	26	30
Servais, 2018[339]	33	58 (5-69)	BM/multiple	NRe (1.00-4.00)	1 (1-2)	22	41	37
Lucchini, 2012[332]	11	NM	BM/multiple	1.00	NRe	18	36	46
Perez-Simon, 2011[333]	10	37 (21-62)	BM/multiple	1.00-2.00	NRe (1-2)	10	60	30
Herrmann, 2012[334]	12	48.5 (21-61)	BM/16	1.70-2.30	2 (2-19)	58	33	9
vonDalowski, 2016[338]	58	55 (19-71)	BM/multiple	0.99 (0.45-2.08)	2 (1-6)	9	38	53
Dotoli, 2017[345]	46	28 (1-72)	BM/NRe	6.81 (0.98-29.78)**	3 (1-7)	7	43	50
Bader, 2018[340]	69	8.2 (0.5-18) 45.5 (18.9-65.5)	BM/pooled	NRe (1.00-2.00)	NRe (1-4)	32	51	14*
Introna, 2014[341]	37	27.8 (1-65)	BM/NRe	NRe (0.80-3.10)	NRe (2-11)	30	43	27

Salmenniemi, 2017[343]	26	8 (12-14) 45 (21-66)	BM/NRe	2.00 (1.40-2.70)	5 (1-6)	27	34	39
Resnick, 2013[344]	50	19 (1-69)	BM/multiple	1.00 (0.3-3.10)	NRe (1-4)	34	32	34
Zhao, 2015[242]	28	26 (14-54)	BM/multiple	4.00 (2.00-8.00)	1 (NRe)	37	29	34
Ringden, 2018[347]	17	54.5 (0.9-65.6)	DC/NRe	2.00 (0.90-2.80)	1 (1-5)	30	29	41
Ringden, 2018[347]	21	48.9 (1.6-72.4)	DC/NRe	1.20 (0.90-2.90)	2 (1-6)	52	48	0

Table 1.1. MSC use in aGvHD: characteristics of each study.

BM: Bone Marrow. **CR:** Complete Response. **DC**: Decidual Cells. **NR**: No Response. **NRe**: Not reported. **PR**: Partial Response. **UC**: Umbilical Cord. *: 3%, no data available at day 28. ******: cumulative dose.

1.2.5.2 The role of MSCs for the treatment of cGvHD

Therapeutic options in cGvHD are limited and mainly based on the use of steroids as first line. Results are disappointing, because of the significant toxicity due to the prolonged use of systemic corticosteroids. Furthermore, 50%-60% of the patients require a second immunosuppressive agent[354]. Although several treatments have been used as second line, none of them has proved its efficacy, thus consensus on how two manage resistant patients is still lacking.

To try to address this unmet need, MSC therapeutic activity has been tested also in cGvHD, albeit the experience in this setting is more limited than in aGvHD. Indeed, most studies reported the treatment of only few patients, and they should be considered as case reports. Results were variable, with overall responses ranging from 0%[341,343] to more than 50% of the patients treated[325,333,334,355]. More promising are the results obtained from larger group of patients and reported in three different studies. In two of these studies a total of 57 steroid-refractory cGvHD were treated with 1 to 5 infusions of BM-MSCs. The median time to response varied between 3 to 24 months after the first MSC infusion[356,357]. Notably, 26% of the patients treated could wean immunosuppressive therapy until complete discontinuation in one of the studies[357]. Recently, 14 patients with moderate to severe cGvHD were prospectively treated with one infusion of AT-MSCs as first-line treatment in association with steroids and cyclosporine[358]. In total 13 patients could be

evaluable, since 1 patient withdrew participation consent. Ten patients achieved a response at 56 weeks (8 complete response [CR] and 2 partial response [PR]), all had stopped steroids and were alive at the end of the study. Conversely, of the three non-responding patients, none was alive and the cause of death was progressive cGvHD[358].

1.2.5.3 MSCs as prophylactic agent against GvHD

MSCs have been demonstrated to enhance haematopoietic engraftment and haematological recovery after both autologous[24] and allogenic[359–361] HSCT when administered at the time of transplant. These findings, along with positive results from preclinical models whereby MSCs were able to delay the onset of GvHD[362,363], prompted investigators to assess whether MSCs could be used prophylactically to decrease the frequency of GvHD when co-administered with the transplant. A comprehensive meta-analysis carried out by Kallekleiv et al[364] determined the potential benefits of MSCs when co-administered with allogenic HSCT within a range of 24 hours (before or after the transplant). The study included a total of 309 patients enrolled in 9 controlled trials performed until May 2015, thereof 3 randomized and 6 non-randomized studies. The analysis suggests that MSCs do not have any beneficial effects in terms of reduction of either aGvHD nor cGvHD, also when patients were sub-categorized into grade I-II or III-IV aGvHD or moderate to severe cGvDH[364]. Important limitation of this meta-analysis relates to the small sample sizes of the studies included and their weak designs, thus

results should be interpreted with caution. However, the evidence provided seems not to support the routinely use of MSCs as GvHD prophylactic agents when co-transplanted with HSCs.

These data are in line with the notion of “licensing” of the MSC immunosuppressive properties already described in the paragraph dedicated to the immunobiology of MSCs. This concept finds support not only *in vitro* but also in preclinical models of GvHD, whereby MSC therapeutic activity could be obtained only when MSCs were infused in the presence of a specific inflammatory milieu[52]. Accordingly, MSCs were effective in reducing GvHD signs only when multiple infusions were administered after transplant but not when one single dose was co-infused with HSCT[365]. These experimental observations have been strongly supported by a meta-analysis recently performed by Wang and collaborators[366]. In this work, the authors extended the previously cited meta-analysis performed by Kallekleiv[364] with three larger randomized clinical trials. The research was performed until January 2018 and included 6 randomized clinical trials enrolling 365 patients. MSCs were infused at different time points from HSCT (within 24 hours, at a median time of 28 days, or with multiple infusions at different time points). The analysis showed that infusion of MSCs significantly reduced the incidence of cGvHD and there was a trend of longer OS in MSC-treated patients[366]. Importantly, the meta-analysis on different sub-groups demonstrated that these favourable outcomes were significantly associated with late MSC administrations, thus supporting a more effective role of MSCs when licensed by a specific microenvironment developed after HSCT.

1.2.6 Summary and future challenges in MSC-based therapy of GvHD

MSCs have very potent immunosuppressive activity against any cell of the immune system.

Their immunobiology makes them ideal candidates for their use in cellular therapy in several immune mediated diseases, including GvHD[1]. The MSC ability to reduce GvHD is conserved across MHC barriers[367]. This observation is confirmed by clinical data in humans, whereby the use of MHC-matched, haploidentical or third-party MSC donors does not have any impact on patient outcomes (see previous paragraph 1.2.5, and Table 1.1).

After thousands of infusions the most convincing conclusion is that MSC infusions are well-tolerated. Major infectious events or disease relapse do not seem to increase after MSC therapy[351,366]. Furthermore, responding patients, who presented complete disappearance of GvHD symptoms or a reduction of their GvHD clinical scores, survive much longer than those patients presenting stability or progression of GvHD after MSC therapy (thus defined as non-responders). Notably, achievement of a response is associated with a predicted OS at 2 years of more than 50%, remarkable result if the poor prognosis of the disease is taken into account.

Despite these very encouraging results, conclusive proof of efficacy has not yet been provided, and the only randomised phase III trial, making use of commercially available MSCs (Prochymal, Osiris), missed its endpoint (improvement of CR at day 28 after MSC infusion in comparison with placebo treated patients). However, this failure was only announced by press-release and results never published in a peer-reviewed manuscript. Intense arguments on the efficacy of MSCs in GvHD has been ignited in the last few years[368], and the recent clinical commissioning policy on GvHD treatments published by NHS England concluded that there was not enough evidence for supporting the use of MSCs in GvHD patients[369].

These considerations highlight an unmet need to better understand how to improve the durability and the rates of responses to MSCs in GvHD patients. Insofar, any step forward has been undermined by our poor knowledge of how MSCs deliver their therapeutic activity. As described in this Chapter, many mechanisms have been described. However, none of them have been translated into effective tools which can be implemented to ameliorate clinical outcomes of the patients treated. This aim can be achieved only by moving back from bed to the bench and by the reconsideration of the mechanisms employed by MSCs to deliver their therapeutic properties *in vivo*[32,370]. Filling the gaps in our understanding on how MSCs actually mediate immunosuppression *in vivo* will translate into the discovery of effective potency assays or biomarkers which will guide patients' stratification and help the prediction of their clinical outcomes. Importantly, the implementation of these biomarkers will guide investigators toward well-designed prospective

phase III clinical trials, with whom robustly and definitely assessing where MSCs should be placed in the GvHD therapy armamentarium.

Furthermore, only unravelling these mechanisms will allow the identification of the necessary tools to harmonize the broad heterogeneity among MSC manufacturing processes across different centres[371], which is a crucial pre-requisition for rigorous and scientifically acceptable comparison between different MSC preparations and for reproducibility across studies.

1.2.7 General hypothesis

We hypothesise that crucial steps for improving the use of MSCs for the treatment of GvHD are the following:

1. Decipher how MSCs deliver their therapeutic activity *in vivo* early after infusion, starting from the observation that MSCs do not engraft in the recipient.
2. Identify mechanistic biomarkers which can be used to predict the clinical outcomes of the patients

1.2.8 Key objectives

To address these hypotheses, we have selected to investigate:

1. The fate of MSCs after infusion. In details, we tested whether MSCs undergo apoptosis after administration.

2. The role of apoptosis in the therapeutic activity of MSCs. In particular, we tested whether the apoptosis hampered MSC efficacy or was instrumental in the immunosuppressive activity of MSCs.

3. The factors associated with the induction of MSCs apoptosis *in vivo*.

4. The validity of these factors for the identification of suitable biomarkers able to predict the clinical outcome of GvHD patients. In details, the biomarker will be tested in a cohort of patients treated with MSCs, and its association with the clinical response assessed after one week from MSC infusion.

5. The validity of the assessment of the response at one week after MSC infusion as predictor of treatment failure/success. This will be tested by analysing the overall survival times of aGvHD patients treated with MSCs and classified as responders and non-responders according to this early assessment.

2 Material and Methods

2.1 Mice and disease models

C57BL/6 (H2b) and Balb/C (H2d) mice were purchased from Harlan Laboratories. Matahari (Mh) (C57Bl/6 background, $CD8^+$Tg, H-2b, $CD45.2^+$, H-2Db-restricted)[372] mice are transgenic for a TCR specific for the male antigen UTY presented in the context of H-Db. Mice were bred in-house and maintained at the Biological Service Unit of the Royal Free and University College London Medical School (London, UK) and of Charles River UK Ltd. All mice were used between 6 and 12 weeks of age.

aGvHD was induced as previously described[373]. Briefly, after lethal irradiation (11 Gy), recipient C57BL/6 male mice were transplanted with $1x10^6$ purified $CD8^+$ T cells from female Mh mice, $5x10^6$ unfractionated BM and $2x10^6$ purified $CD4^+$ T cells from female C57BL/6 wild-type donors. The control group received BM and purified $CD4^+$ cells only. $CD4^+$ and $CD8^+$ T cells were obtained by positive selection using magnetic beads (Miltenyi Biotec Ltd). Live human MSCs ($1x10^6$) were injected intravenously (i.v.) at day +3, whilst MSCs made apoptotic *in vitro* (ApoMSCs) ($1x10^6$) were administered i.v. or intraperitoneally (i.p.) at day +1, +3 and +6 from the transplant. Unless otherwise specified, animals were euthanized for analysis at day +7. The infiltration of GvHD effector cells was assessed by flow-cytometry and the

percentage was expressed as proportion of cells in the lymphocyte gate, based on the physical characteristics of the cells.

For the depletion of all phagocytes mice received 1 mg liposome clodronate (ClodronateLiposomes.com) i.v. 72 hours before MSC infusion[374]. Recipient IDO activity was inhibited by using 1-methyl-D-tryptophan (1-DMT) treatment (Sigma-Aldrich Company Ltd) (2mg/ml) in the drinking water starting from 6 days prior to MSC injection until animals were sacrificed[375].
C57BL/6-Prf1tm1Sdz/J (Perforin$^{-/-}$) mice were purchased from Jackson labs, bred with Matahari Rag2$^{-/-}$ mice and the resulting offspring intercrossed for 2 generations to obtain Mh Rag2$^{-/-}$/Perforin $^{-/-}$ F3 mice.

OVA-induced airway inflammation was induced as previously described[376]. Briefly, female Balb/C mice (Harlan Laboratories) were injected i.p. with 30 µg of chicken egg albumin (OVA type V) (Sigma-Aldrich Company Ltd) on day 0 and 7. Controls received vehicle (aluminum hydroxide) only. On day 14, 15 and 16 animals were challenged with an aerosolized solution of OVA (3%) for 25 minutes. MSCs or ApoMSCs were injected 1 hour after the last challenge. After additional 18 hours, mice were terminally anaesthetized with urethane (2 g/kg i.p.) (Sigma-Aldrich Company Ltd), a cannula inserted into the exposed trachea and three 0.5 mL aliquots of sterile saline were injected into the lungs. The total number of cells in the lavage fluid was counted. For differential cell counts, cytospin preparations were stained with Diff Quick (DADE Behring) and cells counted using standard morphological criteria.

In all experiments, animals were randomly allocated to control or experimental groups. No blinding approach was adopted. Animal procedures were carried out in compliance with the UK Home Office Animals (Scientific Procedures) Act of 1986.

2.2 Cell preparations and media

Cultures were carried out in complete Roswell Park Memorial Institute medium (RPMI) 1640 medium containing GlutaMAX^{TM}, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (25mM), Penicillin 5000 U/ml and Streptomycin 5000 µg/ml (ThermoFisher Scientific), foetal bovine serum 10% (Labtech.com).

Human peripheral blood samples from healthy donors were procured by the National Blood Service (Colindale, UK) as leukocyte cones. Samples from GvHD patients were collected within 24 hours before MSC injection. Informed consent was obtained in accordance with the local ethics committee requirements. PBMCs were isolated by density gradient separation on Histopaque-1077 (Sigma-Aldrich Company Ltd,).

Murine splenocytes were isolated through a cell strainer (BD Falcon), whilst lungs were cut into small pieces and incubated with Collagenase type IV (250 U/ml) (Lorne Laboratories), deoxyribonuclease I from bovine pancreas (250 U/ml) (Merk Millipore) and foetal bovine serum 6.25% at 37ºC for 1 hour.

2.3 MSC preparations

Clinical grade BM-derived human MSCs were generated from BM aspirates collected from the iliac crest of 2 healthy donors. Briefly, 2 ml of BM aspirate were collected in a tube with 100 µl preservative-free heparin. The cells were plated within 24 hours at a density of 10-25 million/636 cm^2 by using alpha modified Eagle's medium (ThermoFisher Scientific), conservative-free heparin (1 UI/ml) (Wockhardt UK Limited) and 5% platelet lysate and then incubated for 3 days at 37 °C and 5% CO_2 ambience. Non-adherent cells were discarded by washing with phosphate buffered saline (ThermoFisher Scientific). When cell confluence of 90-100% was achieved, cells were detached with Trypsin- Ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin, 0.5γ mM EDTA•4Na) (ThermoFisher Scientific) and reseeded at a density of 5000 cells/cm^2. MSCs were used at passage 2 for all *in vivo* experiments, whilst they were used by passage 8 for the in vitro experiments. In the latter case we did not observe any difference in terms of apoptosis susceptibility between different passages. Released criteria were based on positivity (>80%) for CD105, CD90, CD73, negativity (<2%) for CD3, CD14, CD19, CD31, CD45. In each experiment, MSCs were derived from a single expansion and not pooled.

MSCs used for the treatment of the patients enrolled in this study were manufactured at Imperial College Healthcare NHS Trust or at the University

Hospital Carl Gustav Carus, Dresden for those patients treated in the UK or Germany, respectively.

ApoMSCs were obtained by plating $5x10^5$ cells per well in a 96 round-bottom well plate in the presence of synthetic human Granzyme B (GrB) (5 µg/ml) (Enzo Life Sciences) and anti-FAS human (activating, clone CH11) (10 µg/ml) (Merk Millipore) for 24 hours in complete RPMI. The concentration of GrB and FasL was chosen to produce at least 80% of MSC apoptosis.

2.4 Patient details

For the study of the cytotoxic assay, a cohort of 32 patients affected by steroid resistant GvHD was studied. Patients were enrolled between November 2012 and December 2017, they were treated with MSCs in the Department of Haematology at Imperial College London, Southampton University Hospital, Bristol Haematology and Oncology Centre, Manchester University Hospital, and the University Hospital Carl Gustav Carus, Dresden. MSCs were administered for compassionate use (according to Regulation (EC) No 1394/2007). The diagnosis of GvHD was made on histological criteria and GvHD staged according to standard criteria[377,378]. Patients were considered to be steroid-refractory if: (a) those with aGVHD failed to respond to high-dose methylprednisolone after 6 days; (b) the one with cGVHD failed to respond to high-dose steroids after 2-4 weeks, with the addition of Mycophenolate Mofetil (MMF) and cyclosporine (CSA) at 1 and 4 weeks

respectively. Clinical responses to MSCs were assessed 1 week after MSC infusion and defined as an improvement of at least 50% in at least one organ affected by GvHD. Patient characteristics are summarized in Table 3.1.

To verify the clinical validity of the assessment of the response to MSCs after 1 week from infusion, the retrospective analysis of the data of a cohort of 60 patients was carried out. Of these patients, three were included in the study of the cytotoxic assay.

The patients included in this analysis were affected by steroid resistant aGvHD and treated with BM-MSCs between May 2008 and December 2014 at the following UK centres: King's College Hospital NHS Trust, Imperial College Healthcare NHS Trust, London, Central Manchester University Hospital, Manchester, University Hospitals Bristol, Bristol, University Hospital Southampton, Southampton, Birmingham Women's and Children's Hospitals, Birmingham, The Christie NHS Foundation Trust, Manchester, London, Royal Hallamshire Hospital, Sheffield, Sheffield Children's Hospital, Sheffield, Heart of England NHS Foundation Trust, Birmingham and Plymouth Hospitals NHS Trust, Plymouth. Patient characteristics are summarized in Table 5.1.

2.5 Imaging of MSCs

MSCs were transfected using electroporation (Gene Pulser Xcell, BioRad). Cells were suspended in a total volume of 250 µl of buffer and electroporated in 0.4 cm gap cuvettes using 10 µg of DNA at 250 volts and 950 F. Vectors used for transfection were the pGL3-Control vector containing the SV40

promoter for the expression of *Luc*+ (Promega) or the pECFP-DEVDR-Venus (Addgene). When pECFP-DEVDR-Venus was used, the donor fluorophore pECFP and the acceptor Venus-YFP were linked through the flexible linker DEVDR which is recognized and cleaved by the active form of caspase 3. In this system, caspase 3 activity can be monitored through the analysis of the Förster Resonance Energy Transfer (FRET) between pECFP and Venus-YFP. When caspase 3 is not active, the flexible linker DEVDR remains intact and energy transfer from pECFP is allowed with emission of YFP signal. Conversely, in the presence of caspase 3 activation DEVDR is cleaved, thus energy transfer is lost and the pECFP signal increases.

For confocal imaging, pECFP-DEVDR-Venus transfected MSCs were plated in complete RPMI at a concentration of $1x10^5$ cells in a 30 mm x 10 mm dish (Corning) and let adhere overnight. The following day PHA-aPBMCs were added at a PBMC:MSC ratio of 40:1. Where indicated, pan caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (Z-VAD-FMK) (50 µM), perforin inhibitor ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) (4 mM), GrB-inhibitor methyl-5-chloro-4-oxo-3-[2-[2-(phenylmethoxycarbonylamino) propanoylamino] propanoylamino] pentanoate (Z-AAD-CMK) (300 µM) were used. Living cell imaging was acquired every 3 minutes for 180 minutes using a Leica TCS-SP5 II Confocal Microscope, with 488 nm and 407 nm lasers. The images were processed and analyzed by using the software “R” and EBImage package.

In vivo imaging was performed injecting i.v. $1x10^6$ MSCs transfected with luciferase (luc-MSCs) into naïve C57BL/6, BM or GvHD mice 3 days after the transplant in the GvHD model. In the airway inflammation model, luc-MSCs were infused i.v. in naïve Balb/C or OVA-treated mice 1 hour after the last OVA challenge. After one additional hour, mice were anesthetized with isoflurane (1.5% isofluorane, 98.5% Oxygen), injected i.p. with 3 mg of VivoGlo Casp 3/7 substrate Z-DEVD Aminoluciferine (Promega,) and imaged using IVIS® Lumina III (PerkinElmer) system for a total time of 5 minutes. Images were analyzed by using the software "R" and EBImage package to obtain mean total luminescence signal (TLS). Confirmation of the presence of transfected MSCs was obtained injecting mice with VivoGlo Luciferin (Promega) 30 minutes after the administration of substrate Z-DEVD Aminoluciferine.

2.6 Detection of efferocytosis

MSCs were first labelled using CellTrace Violet labelling (ThermoFisher Scientific) at a final concentration of 5 µM and then made apoptotic (ApoMSCs) as described above, using synthetic human GrB (5 µg/ml) and anti-FAS human (10 µg/ml) for 24 hours. $10x10^6$ labelled apoMSCs were then injected i.p. or i.v. and mice sacrificed after 2 hours post-injection. Spleen, lungs, peritracheal, paratracheal, pericardial, mesenteric, periportal and celiac lymph nodes were collected and analysed by flow-cytometry. Positivity of CellTrace Violet was assessed as measure of ApoMSC engulfment in

$CD11b^+$ and $CD11c^+$ gated subpopulations of phagocytic cells. Cells positive for the CellTrace Violet were then assessed for their expression of IDO.

2.7 Pre-activation of human PBMCs and murine $CD8^+$ cells

PBMCs pre-activated with phytohemagglutinin (PHA) (PHA-aPBMCs) were obtained plating $5x10^6$ human PBMCs in 24-well plate in the presence of PHA (5 µg/ml) (Sigma-Aldrich Company Ltd) in a final volume of 2 ml of complete RPMI for 72 hours. PBMCs pre-activated with MLR (MLR-aPBMCs) were obtained using one-way MLR in which PBMCs from one donor (stimulators) were irradiated (30 Gy) and co-cultured with the PBMCs of an unrelated donor (responder) at a stimulator:responder ratio of 1:1 in complete medium at a density of $0.75x10^6$ cells/cm^2. Cells were then incubated at 37º C, 5% CO_2 for 5 days.

NK cells were purified by positively selecting $CD56^+$ cells from healthy donor PBMCs (Miltenyi Biotec Ltd) and activated with recombinant human-IL-2 (1000 U/ml) (Peprotec EC Ltd).

New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1)-specific $CD8^+$ T cell clone (Clone 4D8) was kindly supplied by Prof. Vincenzo Cerundolo (Institute of Molecular Medicine, Oxford university, UK). The clone was expanded in complete RPMI 1640 with Sodium Pyruvate (1 mM), 2-Mercaptoethanol (0.05 mM) (ThermoFisher Scientific), recombinant human-IL-2 (400 U/ml) (Peprotec EC Ltd) and PHA (5 µg/ml) (Sigma-Aldrich Company Ltd)[379].

Mh $CD8^+$ cells were stimulated using the following protocol: $5x10^6$ purified Mh $CD8^+$ cells were plated in 24-well plates in the presence of CD3/CD28-coated beads (Dynabeads®) (ThermoFisher Scientific) in a final volume of 2 ml of complete RPMI and incubated for 72 hours.

2.8 Immunosuppressive assay

Serial dilutions of human MSCs were plated in a flat bottom 96-well plate and let adhere overnight in 100 µl of complete RPMI. Where indicated, MSC cultures were exposed to human Interferon-γ (hIFNγ) and human TNF-α (hTNFα), murine IFN-γ (mIFNγ) and murine TNFα (mTNFα) (20 ng/ml each) (all cytokines were from Peprotec EC Ltd), supernatant from PHA-aPBMCs or from murine splenocytes (mSpl) activated with concanavalin A (ConA). Both supernatants were obtained from 72-hour stimulation of human PBMCs or mSpl with 5µg/ml (PHA) or 3µg/ml (ConA), respectively.

The following day, $5x10^5$ Balb/C mSpl were labelled with Carboxyfluorescein Diacetate Succinimidyl Ester dye (ThermoFisher Scientific) and plated with MSCs at escalating MSC:mSpl ratios. Culture controls consisted of mSpl plated without MSCs in the presence (positive control) or in the absence (negative control) of ConA (3µg/ml). Proliferation of mSpl was then assessed by flow-cytometry after 72 hours and expressed as the percentage of the proliferation obtained at each MSC:mSpl dilution in comparison with the one obtained in the positive control culture. Results were expressed as percentage of inhibition.

2.9 Cytotoxic Assay

$1x10^5$ MSCs were plated overnight in a total volume of 500 µl. The day after pre-activated immune cells were plated at different concentrations (2.5 to 40:1 effector:MSC ratios). MSC apoptosis was then tested at different time points using flow-cytometry or confocal microscopy analysis. Eventually, the assay was performed for 4 hours in most of the cases. At flow-cytometry, MSCs were identified as $CD45^-$ cells.

Antigen-specific cytotoxic activity of clone 4D8 was tested using T2 cells pulsed with NY-ESO-1 antigen (epitope SLLMWITQC) at a concentration of 0.1 µM for 1 hour. In the competition assay, T2 (from Hans Stauss, University College London) and K562 cells (from Junia Melo, Imperial College London) were discriminated from effector cells by CellTrace Violet labelling. The tracer concentration was optimized for the T2 (1 µM) and K562 (2.5 µM) cells. Cell lines were tested for mycoplasma contamination before use.

When flow-cytometry was used, the level of apoptosis was assessed using the PE annexin-V apoptosis detection kit (BD Biosciences). Unless specified, apoptotic cells were identified as annexin-V^+/7-AAD^- cells.

2.10 Inhibitors

Cultures were supplemented with pan-caspase inhibitor Z-VAD-FMK (10 µM in the flow-cytometry experiments or 50 µM in the living cell confocal experiments) (R&D System), perforin inhibitor EGTA (4 mM) (Sigma-Aldrich Company Ltd), GrB inhibitor Z-AAD-CMK (300 µM) (Merk Millipore), AMD3100 (antagonist of CXCR4, for blockage of the CXCL12/CXCR4 axis) (25 µg/ml, and 250 µg/ml) (Sigma), neutralizing antibodies against HLA-DR (clone L243) (50 µg/ml), human HLA-A,B,C (clone W6/32) (100 µg/ml) (BD Biosciences), TNFα antagonist Etanercept (Enbrel®) (10 µg/ml or 100 µg/ml) (Amgen), CD18 (forming the β2 integrins lymphocyte function-associated antigen-1 and macrophage-1 antigen) (Clone TS1/18, 9 µg/ml and 90 µg/ml) (Biolegend), CD29 (Clone P5D2, 10 µg/ml and 100 µg/ml) (R & D Systems), CD44 (Clone Hermes-1, 10 µg/ml and 100 µg/ml) (Thermo Scientific), Intercellular Adhesion Molecule 1 (ICAM-1) (Clone R6.5, 50 µg/ml and 250 µg/ml) (Affymetrix), ICAM-2 (Clone CBR-IC2/2, 50 µg/ml and 250 µg/ml) (eBioscience), vascular cell adhesion molecule 1 (VCAM-1) (Clone P3C4, 100 µg/ml and 250 µg/ml) (Millipore), $\alpha_v\beta_3$ (Clone LM609, 50 µg/ml and 250 µg/ml) (Millipore). Each reagent was incubated with MSCs 1 hour before the culture with effector killer cells. In all cases, the concentration of the corresponding inhibitor was kept for the duration of the cytotoxic assay.

The neutralizing anti-CD178 (Clone NOK-1) (10 µg/ml or 100 µg/ml) (BD Biosciences), anti- TNF-related apoptosis-inducing ligand (TRAIL) (clone 2E2) (10 µg/ml or 100 µg/ml) (Enzo Life Sciences) antibodies, MYR Protein

Kinase-Cζ Pseudosubstrate (PKCζ-PS) (10 µM, 25 µM or 75 µM) (ThermoFisher Scientific) and Etanercept (10 µg/ml or 100 µg/ml) were incubated with effector killer cells for 2 hours before the cultures with MSCs. In all cases, the concentration of the corresponding inhibitor was kept for the duration of the cytotoxic assay.

2.11 Flow-cytometry

The following antibodies specific for murine molecules were used: anti-CD45 (FITC, Clone 30-F11) (eBiosciences Ltd), anti-V_{β}8.3 (FITC, Clone 1B3.3), anti-CD8 (APC, Clone 53-6.7), antiCD4 (PE, Clone H129.19), anti-CD19 (APC-H7, Clone 1D3), anti-NK1.1 (PerCP-Cy5.5, Clone PK136) (BD Biosciences), anti-CD11b (PerCP-Cy5.5, clone M1/70), anti-CD11c (APC-Cy7, clone n418), anti-Ido1 (Alexa Fluo647, clone 2e2) (BioLegend). For human specific molecules, the following antibodies were used: anti-CD45 (FITC, clone 2D1), anti-CD8 (APC, Clone SK1), anti-CD4 (PE, Clone SK3), anti-CD11b (PerCP-Cy5.5, clone M1/70), anti-CD56 (FITC, clone HCD56) (BD Biosciences).

All samples were acquired using BD FACS Canto II using the software FACS Diva and analysed with Flow-jo software. FRET and Caspase activity (CA_f) were assessed by flow-cytometry as previously described[380].

2.12 Real Time quantitative PCR

MSC RNA was obtained from TRIzol® (ThermoFisher Scientific) lysates and extracted using RNeasy Mini Kit (Qiagen). Real Time quantitative PCR (qRT-PCR) was performed following TaqMan® RNA-to-CT 1-Step Kit instructions (ThermoFisher Scientific), using 20 ng of RNA template per reaction. Assays were carried out in duplicates on an StepOnePlus RT PCR system thermal cycler (Applied Biosystem) using TaqMan primers (all purchased from ThermoFisher Scientific). The human primers used were the following: IDO (Hs01589373_m1), TSG-6 (Hs01113602_m1) and Prostaglandin-Endoperoxide Synthase 2 (PTSG2) (Hs00153133_m1) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Hs02800695_m1) as housekeeping gene. Data were then analysed using StepOne software version 2.1 and relative quantification obtained with ΔΔCt method, considering untreated MSCs as reference.

2.13 Statistics

Results were expressed as mean±SD. The unpaired Student *t* test was performed to compare 2 mean values. One-way ANOVA and Tukey's Multiple Comparison test or Hommel post-hoc test was used to compare 3 or more mean values.

In the analysis of the data obtained from the 32 patients with steroid resistant aGvHD used for the study of the cytotoxic assay, the following statistical methods were used: survival curves were given for MSC treated vs untreated patients as well as responders vs non-responders. Median survival times with 95% confidence limits, as well as the p-value from the log rank test were given to compare survival curves. Demographic and treatment characteristics were compared using the t-test or Fisher's exact test. R Version 3.3 and an alpha level of 0.05 were used for all analysis.

The retrospective analysis of the cohort of 60 GvHD patients was performed as following: the associations of patient, disease and treatment factors with survival and disease response were investigated using the log-rank test, chi-squared test or chi-squared trend test, respectively. Probabilities of survival were estimated using the Kaplan-Meier method.

P-values less than 5% ($p<.05$, two-sided) was considered statistically significant. SPSS version 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) was utilized for all calculations.

3 MSC apoptosis after infusion is instrumental for immunosuppression and requires cytotoxic cells to be induced.

3.1 Introduction

In the general introduction, we have described how MSCs exhibit potent immunosuppressive and anti-inflammatory activities. For these reasons, MSCs have received center stage attention and they have been extensively tested in several immune mediated diseases, including GvHD. Despite the very encouraging results, the extensive clinical use has been undeterred by the fact that the mechanisms underlying MSC therapeutic activity remain poorly understood.

Two major unresolved challenges undermine the progress in the field. The first is that, only a proportion of patients, although affected by the same disease, responds to MSC infusions (Table 1.1) and this response cannot be predicted. The second is that the vast majority of infused MSCs become undetectable a few hours after transiently residing in the lungs[2,131]. Despite this, MSCs appear to maintain their ability to deliver therapeutic activities, and our current knowledge cannot provide an explanation to the paradox of the absence of engraftment and immunosuppressive functions[42,59,132,381].

The most obvious explanation for the lack of MSC engraftment is that the cells die after infusion. Understanding whether this impairs or can in fact contribute to the immunosuppressive activity *in vivo* may provide crucial information to improve clinical efficacy and make therapeutic achievements more reproducible. By using a mouse model of aGvHD, we have tested the hypothesis that MSCs undergo *in vivo* apoptosis following exposure to the GvHD environment.

3.2 Results.

3.2.1 MSC undergo apoptosis in recipient GvHD animals.

We utilized a mouse model of GvHD in which lethally irradiated C57BL/6 male mice were transplanted on day 0 with BM cells and polyclonal purified $CD4^+$ T cells from female syngeneic donors and purified $CD8^+$ T cells transgenic for a T-cell receptor specific for the mouse male HY-antigen Uty (Mh) as GvHD effectors[373] (Figure 3.1). The addition of $CD4^+$ T cells is necessary to facilitate the expansion of the $CD8^+$ T cells. In this model, the expansion of the T cells effecting GvHD ($CD8^+V_\beta8.3^+$) can be precisely enumerated and correlates with the clinical severity of the disease.

In order to explain the mechanism by which MSC are rapidly cleared after injection[2,131], we tested the hypothesis that MSC undergo *in vivo* apoptosis. Caspase activation was evaluated by using MSCs transfected with the pGL3 Control Vector containing SV40 promoter and enhancer sequences for the expression of firefly luciferase (*Luc*+) (luc-MSC). Caspase activation was measured by luciferase activity using injection of Z-DEVD-aminoluciferin, a firefly luciferase pro-substrate containing the DEVD oligopeptide recognized by Caspase 3. Only upon activation of Caspase 3, DEVD can be cleaved, aminoluciferin released and eventually metabolized by the firefly luciferase expressed in MSCs. Luc-MSCs were injected into recipients of BM transplant with $CD8^+$ Mh T cells (GvHD group) 3 days after the transplant. One hour

after infusion, caspase activity was measured *in vivo* as TLS. Two groups of control mice received MSCs. One consisted of untreated males (naïve group), and the second was a group of mice which were irradiated and received $CD4^+$ T cells and BM cells (BM group) without the transgenic Mh $CD8^+$ T cells. This latter group reproduces the condition of MSC infusion in the absence of activated cytotoxic GvHD effector T cells. Graphic representation of the aGvHD mouse model and the experimental protocol are summarized in Figure 3.1.

Figure 3.1. Experimental design: GvHD mouse model and Caspase 3 activation study in MSCs.

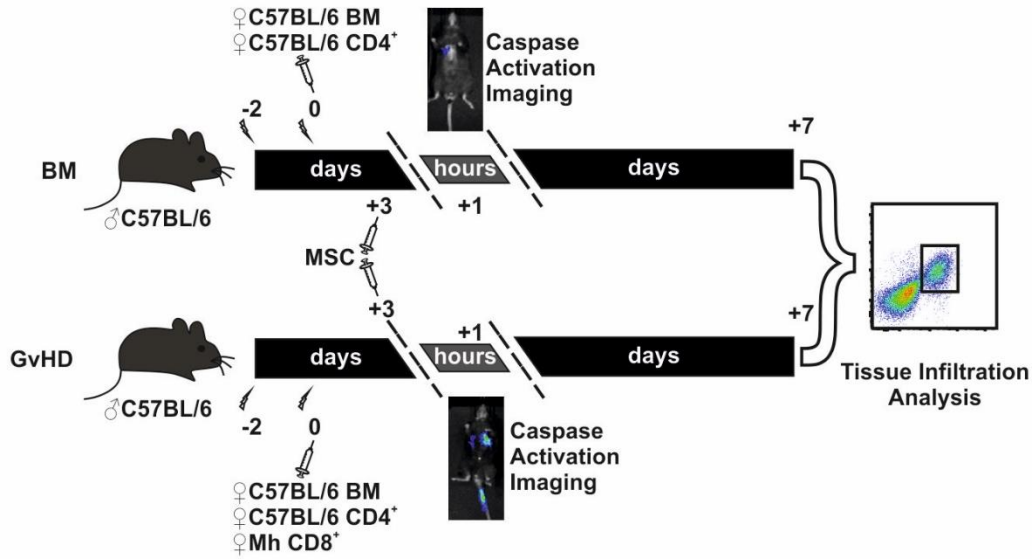

Figure 3.1. Experimental design: GvHD mouse model and Caspase 3 activation study in MSCs Untreated (naïve) or lethally irradiated C57BL/6 male mice were transplanted with bone marrow (BM) and $CD4^+$-purified cells from female syngeneic donors with or without $CD8^+$ cells purified from Mh mice ($CD8^+V_\beta8.3^+$) (GvHD and BM groups, respectively). At day +3 post-transplant, luc-MSCs were infused. One hour after infusion, mice were first anesthetised with Isoflurane, then injected intraperitoneally with Z-DEVD-Amoniluciferin and imaged in an IVIS Lumina Caliper chamber for 5 minutes. At day +7 post-transplant, mice were sacrificed and the infiltration of GvHD effector cells ($CD8^+V_\beta8.3^+$) in lungs and spleen was analysed by flow-cytometry.

High caspase activity, measured as TLS, was observed only in the GvHD group (mean TLS: 26.52 [SD: 12.31, 95% CI: 13.60-39.43]), while significantly lower caspase activity could be found in mice of both BM and naïve groups (mean TLS were 6.13 [SD: 6.72, 95% CI: -0.92-13.19] and 1.86% [SD: 2.98, 95% CI: -1.26-4.99], respectively) (Figure 3.2A and B).

To confirm that luc-MSCs could be tracked in the lungs of all animals, including those with low or absent caspase activity, we subsequently infused mice with the control D-luciferin (firefly luciferase substrate). We could find that high signal could be detected from all animals (Figure 3.2C), with not significant difference among groups. Mean TLS were 29.10 (SD: 13.18, 95% CI: 15.26-42.94), 27.13 (SD: 25.29, 95% CI: 0.594-53.67), and 28 (SD: 6.91, 95% CI: 20.75-35.25) in GvHD, BM and naïve group, respectively (Figure 3.2D.

Figure 3.2. MSCs undergo apoptosis *in vivo* after infusion.

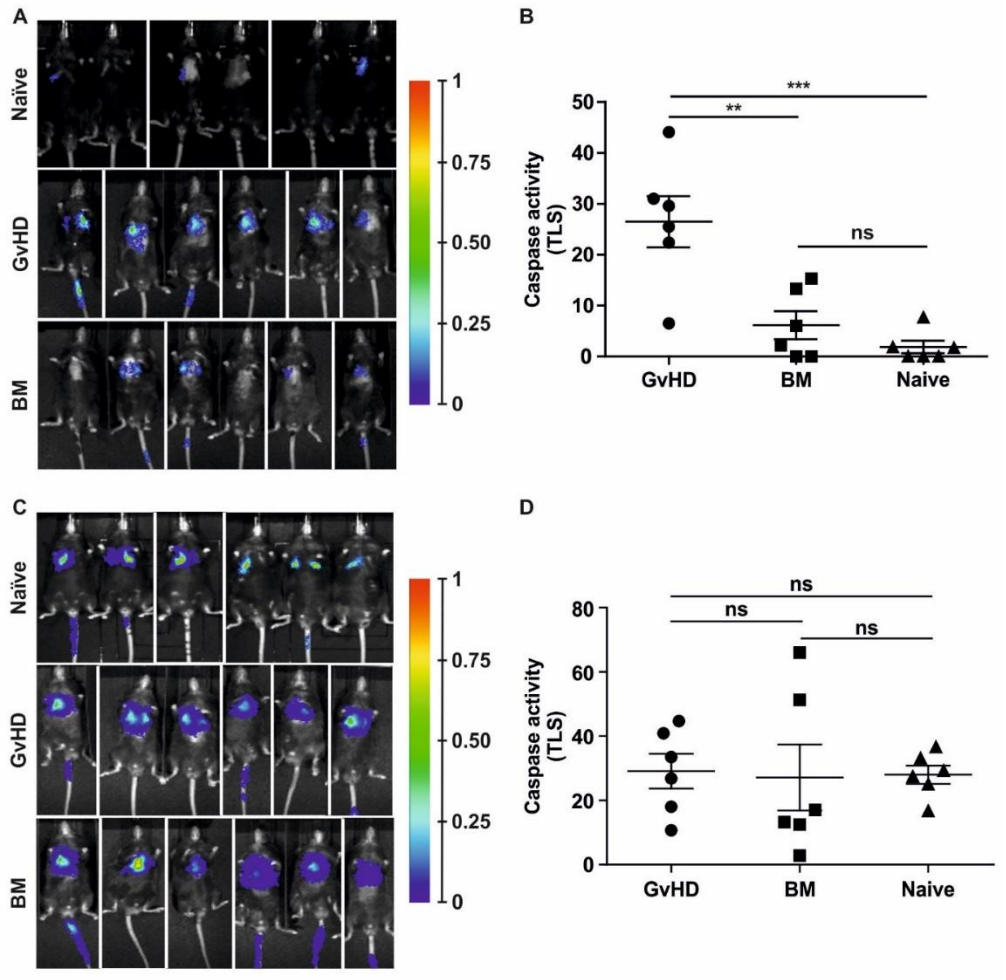

Figure 3.2. MSCs undergo apoptosis *in vivo* after infusion. A: luc-MSCs were injected i.v. into naïve, BM and GvHD mice 3 days after transplantation. All animals were then injected i.p. with DEVD-aminoluciferin and imaged 1 hour later. *N*: total of 6 (1 to 3 mice per group), grouped from 3 independent experiments. In each experiment, a different MSC expansion was used. White lines separate multiple photographs assembled in the final image. **B**: Total luminescence signal (TLS) was measured from the images of mice in Figure 4A and shown as mean±SD. **C:** in order to confirm the presence of luc-MSC

in the lungs of all groups of mice infused with MSCs, the same mice imaged in Figure 4A were injected with D-Luciferin. White lines separate multiple photographs assembled in the final image. **D:** Total luminescence signal (TLS) was measured from the images of mice in C and shown as mean±SD. Statistics in B and D: one-way ANOVA, with Tukey's Multiple Comparison Test. **: p<.01, ***: p<.001, ns: not significant.

The evidence that MSCs undergo apoptosis after infusion prompted the question of whether they were still capable of suppressing antigen-driven T cell expansion. Therefore, we analyzed their immunosuppressive effect by enumerating $CD8^+V_\beta8.3^+$ Mh T cells (GvHD effector cells) in MSC-treated or untreated GvHD mice. MSCs produced a substantial reduction in GvHD effector cell infiltration in both spleen and lungs in comparison with untreated mice (Figure 3.3A-C). Mean percentage of GvHD effector cells was 17.87% (SD: 6.933, 95% CI: 14.03-21.71) and 8.83 (SD: 4.34, 95% CI: 6.20-11.45) in the spleen of untreated or MSC-treated GvHD mice, respectively (Figure 3.3B). Similar results were obtained in lungs, whereby the mean percentage of infiltrating GvHD cells was 19.76% (SD: 6.31, 95% CI: 16.26-23.25) and 12.12 (SD: 2.32, 95% CI: 10.71-13.52) in GvHD and GvHD-MSC mice, respectively (Figure 3.3C). These results indicate that, despite the presence of MSC apoptosis after infusion (Figure 3.2A and B), MSC immunosuppression still occurs.

Figure 3.3. MSCs prevent *in vivo* expansion of GvHD-effector cells in our pre-clinical model of aGvHD.

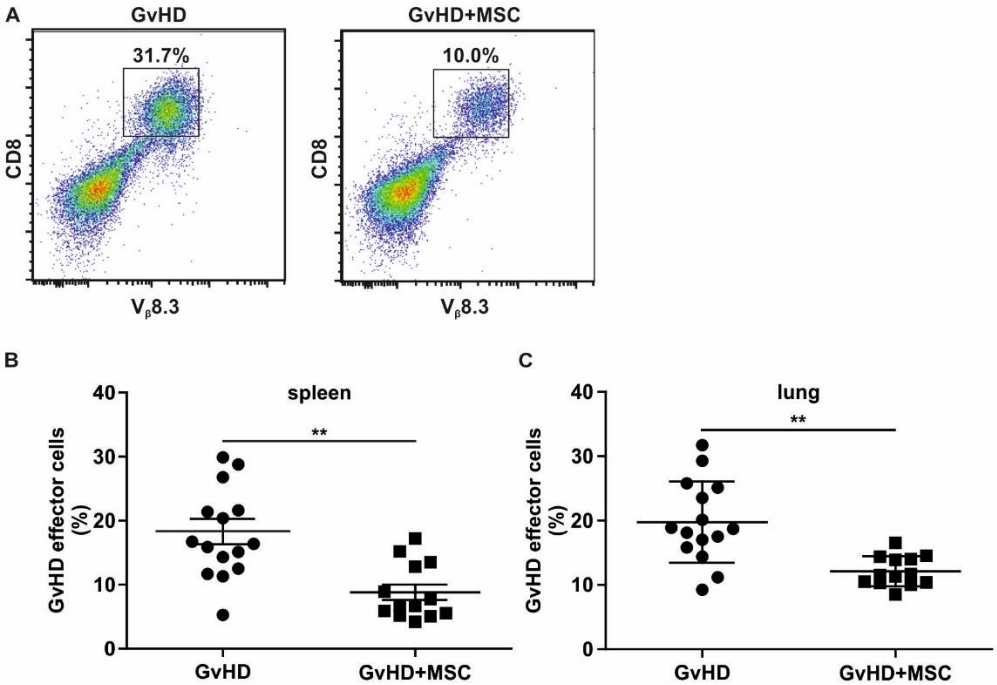

Figure 3.3. MSCs prevent *in vivo* expansion of GvHD-effector cells in our pre-clinical model of aGvHD. A: Percentage of GvHD effector cells ($CD8^+V_\beta8.3^+$) calculated in the lymphocyte gate (defined by the physical characteristics of the cells) in treated (GvHD+MSC) and untreated (GvHD) mice, evaluated 4 days after MSC injection by flow cytometry. Representative dot plot. **B-C:** Infiltration of GvHD effector cells assessed as described in A in the spleen (**B**) and lungs (**C**) of GvHD mice (black circles) and GvHD mice treated with MSC (black squares). *N*: 15 (GvHD) and 13 (GvHD+MSC) mice, grouped from 4 independent experiments; mean±SD are shown. Statistics: unpaired t-test. **: p<.01.

In our model, we could reasonably exclude the possibility that MSC immunosuppressive activity were the consequence of MSC being exposed to the recipient inflammatory cytokines because in our xenogeneic combination murine inflammatory cytokines do not cross-react with the corresponding human receptors and will not activate immunosuppressive molecules in human MSCs[43,82,382], whilst retaining the ability to expand murine effector cells mediating GvHD[383]. Accordingly, human MSCs were not able to inhibit ConA induced proliferation of mSpl unless pre-activated by human cytokines (Figure 3.4A-C). Furthermore, exposure of human MSCs to murine inflammatory cytokines did not upregulate *IDO*, *TSG-6*, or *PTSG2* (enzyme involved in the production of PDGE2), considered major effectors of human MSC-mediated *in vitro* immunosuppression (Figure 3.4D).

Figure 3.4. Human MSC immunosuppression is not 'licensed' by murine cytokines.

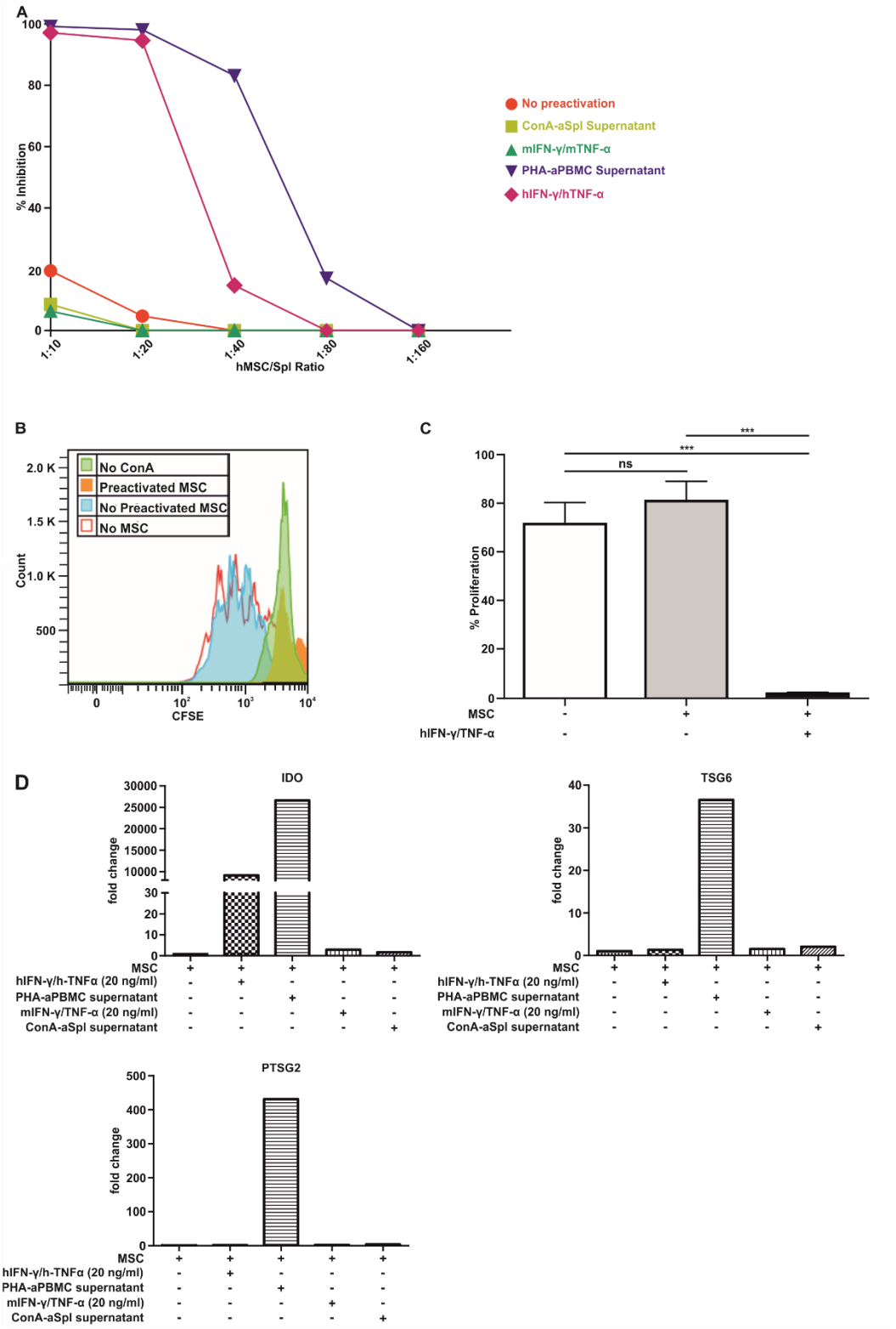

Figure 3.4. Human MSC immunosuppression is not 'licensed' by murine cytokines. A: Human MSCs were plated overnight at serial dilutions alone or with ConA-activated murine Spl supernatant, PHA-activated human PBMC supernatant, murine IFN-γ/TNF-α (20 ng/ml each) or human IFN-γ/human TNF-α (20 ng/ml each) as indicated. MSCs were then tested for the ability to inhibit ConA-induced splenocytes proliferation for 72 hours. Proliferation was determined by carboxyfluorescein succinimidyl ester staining. The percentage of inhibition was calculated subtracting the percentage of proliferation to 100 and then plotting against the corresponding MSC/Spl ratio. **B:** human MSCs were plated overnight either untreated or exposed to hIFNγ/hTNFα (20 ng/ml each) as indicated and then tested for the ability to suppress mSpl proliferation at 1:10 MSC/Spl ratio. Representative histogram plot. **C:** human MSCs were plated as in B. Results of 3 independent experiments are shown. **D:** human MSCs were incubated for 24 hours with PHA-stimulated human PBMC or ConA-stimulated murine Spl (separated by a transwell), mIFN-γ/mTNF-α (20 ng/ml each) or hIFN-γ/hTNF-α (20 ng/ml each) as indicated. *IDO, TSG-6*, and *PTSG2* expression were then assessed by real time PCR and calculated as relative expression in comparison to that of untreated MSCs. Representative results of three independent experiments are shown. Statistics: One-way ANOVA and Tukey's Multiple Comparison test. ***: p<.001. MSC: Mesenchymal Stromal Cells, ConA: Concanavalin-A, Spl: splenocytes, PHA: phytohemagglutinin, PBMC: Peripheral Blood Mononuclear Cells, IFN-γ: Interferon-γ, TNF-α: Tumour Necrosis Factor-α.

3.2.2 In vivo MSC apoptosis depends on activated recipient GvHD effector cells.

Our results show that MSCs rapidly undergo apoptosis after infusion, providing an explanation for the rapid clearance of transplanted MSCs in the recipient[2,131]. The absence of *in vivo* MSC apoptosis in naïve mice clearly demonstrates that MSC apoptosis is not the result of xenogeneic recognition of human MSCs. Furthermore, our results suggest that caspase 3 activation in MSCs is associated with the presence of cytotoxic cells, since MSC apoptosis could be detected only in GvHD but not in BM mice (mice not receiving GvHD effector cells in their transplant) (Figure 3.2A and B).

To better evaluate this relationship, we first enumerated GvHD effector cell infiltrate ($CD8^+V_\beta8.3^+$) in the lungs of mice, where MSC apoptosis occurs in all groups. We found that only the lungs of GvHD, but not naïve and BM mice, contained a large proportion of $CD8^+V_\beta8.3^+$ cells (Figure 3.5A), thus confirming the correlation between caspase activation in MSCs and the presence of GvHD effector cells. Percentage of $CD8^+V_\beta8.3^+$ cells in lungs was 0.01% (SD: 0.03, 95% CI: 0.01-0.17), 0.59% (SD: 0.76, 95% CI: -1.29-2.48), and 14.22% (SD: 3.75, 95% CI: 11.83-16.60) in naïve, BM, and GvHD mice, respectively.

To test the hypothesis that GvHD effector cells were responsible for MSC apoptosis, MSCs were cultivated with $CD8^+$ T cells purified from the lungs or spleens of GvHD (*in vivo* activated) or naïve Mh (*in vivo* resting) mice. Activated, but not resting, Mh $CD8^+$ T cells induced MSC apoptosis (Figure

3.5B). Indeed, percentage of annexin V^+/7-AAD^- cells were 19.37% (SD: 0.35, 95% CI: 18.49-20.24) in MSCs when in co-culture with $CD8^+$ T-cells from lungs obtained from naïve mice and 30.63% (SD: 5.88, 95% CI: 16.02-45.25) in MSCs when in co-culture with $CD8^+$ T-cells from lungs obtained from GvHD mice. Similarly, apoptotic MSCs were 11.95% (SD: 0.63, 95% CI: 6.23-17.67) when in co-culture with $CD8^+$ T-cells from spleens obtained from naïve mice, while they were 22.17% (SD: 2.31, 95% CI: 16.42-27.91) in MSCs when in co-culture with $CD8^+$ T-cells from spleens obtained from GvHD. Background level of annexin V^+/7-AAD^- cells in MSCs cultured alone were 11.45% (SD: 4.71, 95% CI: 6.50-16.40) (Figure 3.5B). Similar proportion of cytotoxicity against MSCs could be elicited by naïve Mh $CD8^+$ T cells when stimulated *in vitro* by CD3/CD28 beads (Figure 3.5C). Annexin V^+ cells were 11.13% (SD: 0.83, 95% CI: 3.63-18.63) and 39.20% (SD: 0.84, 95% CI: 31.58-46.82) in MSCs alone and MSCs in co-culture with CD3/CD28 activated $CD8^+$ T-cells, respectively.

Taken together, these data show the association between the presence of activated GvHD effector cells in MSC-recipient mice and induction of MSC apoptosis after infusion.

Figure 3.5. MSC apoptosis is associated with the presence of activated GvHD effector cells.

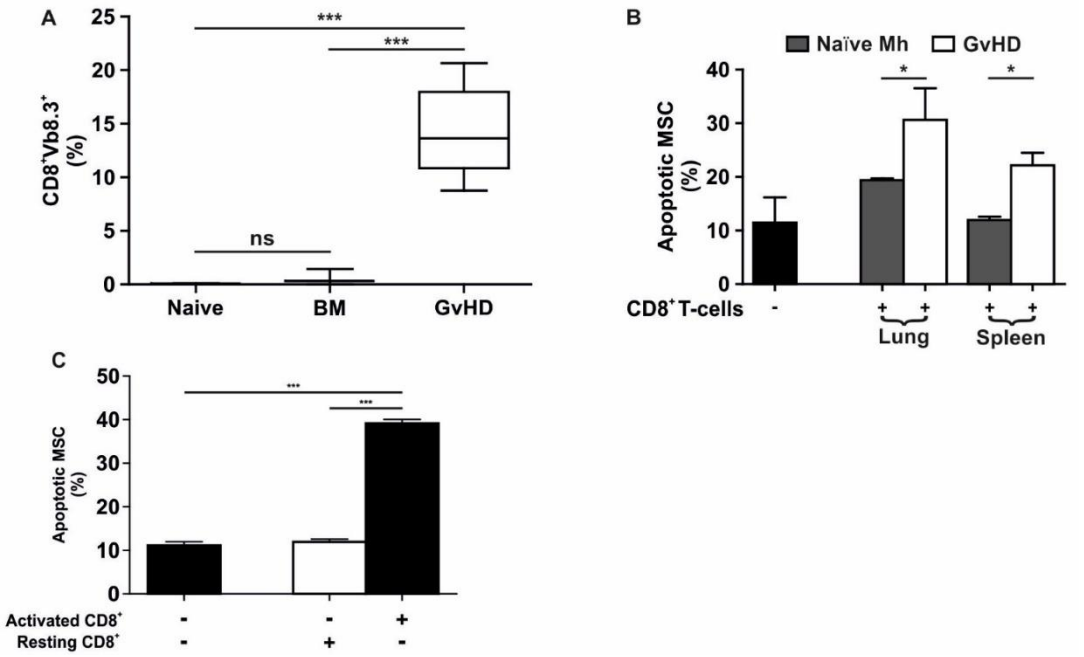

Figure 3.5. MSC apoptosis is associated with the presence of activated GvHD effector cells. **A:** The percentage of $CD8^+V_\beta8.3^+$ cells in lung cell suspensions from naïve C57BL/6 male, BM or GvHD mice was analyzed in the lymphocyte population; mean±SD are shown. N: 12 (GvHD), 3 (BM) and 3 (naïve) mice, grouped from 3 independent experiments. **B:** $CD8^+$ cells were sorted from the lungs and spleens of naïve female Mh (grey bars) or GvHD mice (not treated with MSC) (white bars) 7 days after the transplant and tested for their ability to induce MSC apoptosis *in vitro*. The results show annexin-V^+/7-AAD^- MSC (mean±SD) in 3 independent experiments (N=10 per group), black bar represents the value of apoptosis in MSCs cultured alone used as control (N: 3). **C:** $CD8^+$ T cells isolated from naïve female Mh mice were stimulated for 3 days with anti-CD3/CD28 beads and cultured with

MSCs at a 20:1 Mh T-cell:MSC ratio. After 4 hours apoptosis was assessed in MSC by annexin-V/7AAD staining. Results represent the mean±SD of 3 independent experiments. Statistics: One-way ANOVA, with Tukey's Multiple Comparison Test. *: $p<.05$; ***: $p<.001$.

The requirement of cytotoxic cells in the induction of MSC apoptosis was further evaluated using Mh/Perforin Knock-Out mice (Mh/Perf$^{-/-}$) as donors of defective cytotoxic GvHD effector cells (GvHDPerf$^{-/-}$ group).

Luc-MSCs were infused into GvHDPerf$^{-/-}$ or control GvHD mice which had received Mh CD8^{+} T cells. Mice were imaged 1 hour later, and caspase activation measured as described above. We confirmed that high signal of apoptosis could be observed in the GvHD group (26.07 [SD: 7.40, 95% CI: 19.23-32.92]). However, significantly lower caspase activity could be found in GvHDPerf$^{-/-}$ mice (6.16 [SD: 5.63, 95% CI: 0.95-11.36]) (Figure 3.6A-B).

To assess that the signal detected in lungs of GvHDPerf$^{-/-}$ mice was not dependent on a defective activity of the luciferase vector in MSCs, both groups of mice were eventually imaged using the control D-luciferin. Similar emission patterns were found in both groups, thus confirming that when MSCs were infused into GvHDPerf$^{-/-}$, caspase 3 was not activated (Figure 3.6C-D).

We could conclude that MSC apoptosis *in vivo* requires the presence of functional cytotoxic GvHD effector cells.

Figure 3.6. GvHD effector cells are required to induce MSC apoptosis *in vivo.*

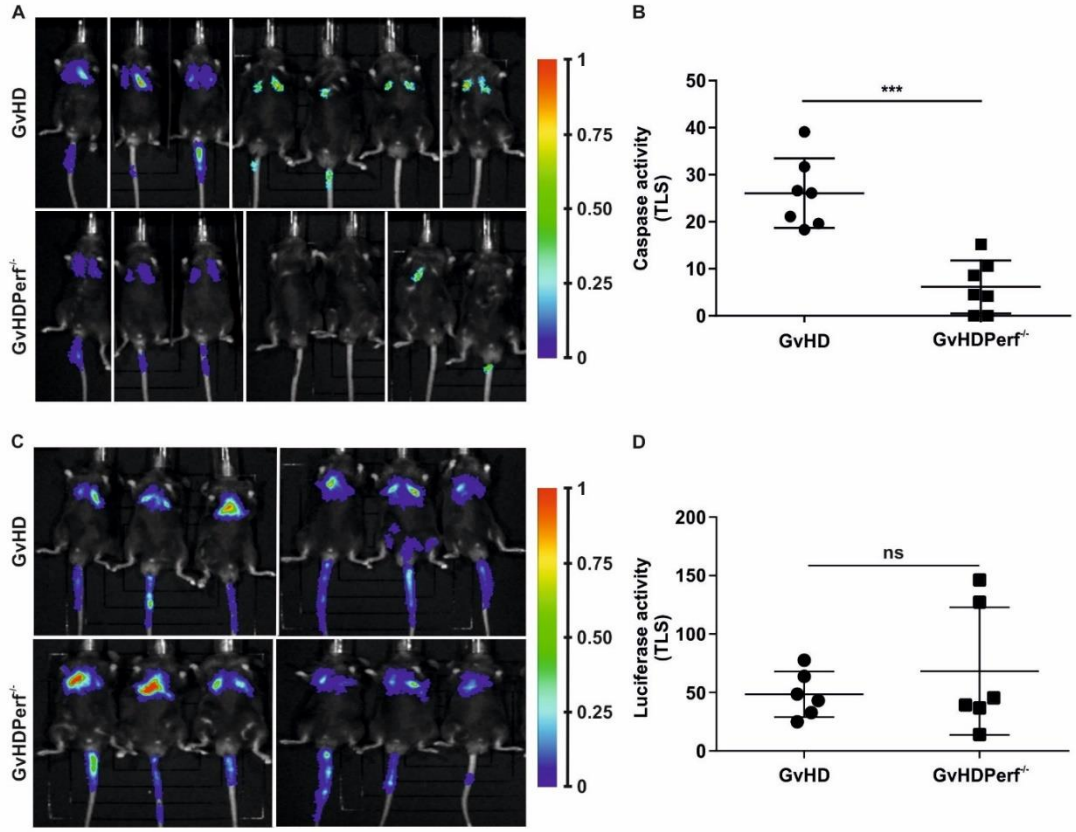

Figure 3.6. GvHD effector cells are required to induce MSC apoptosis *in vivo*. A: luc-MSC were infused in two independent experiments in GvHD (N=7) and GvHDPerf$^{-/-}$ (N=7) mice 3 days after transplantation. 1 hour later mice were injected with Z-DEVD-aminoluciferin and imaged. White lines separate multiple photographs assembled in the final image. **B:** TLS was obtained from Figure 8A and expressed as mean±SD. **C:** In order to confirm the presence of luc-MSC in the lungs of all groups of mice infused with MSCs, the same mice imaged in Figure 2C were injected with D-Luciferin. White lines separate multiple photographs assembled in the final image. **D:** TLS was

measured from the images of mice in Fig. S3B and shown as mean±SD. Statistics: unpaired t-test. ***: p<.001. ns: not significant.

We then evaluated the immunosuppressive activity of MSCs in the absence of apoptosis induction. The infiltration of GvHD effector cells in the spleen and lungs of mice receiving MSCs was not reduced in GvHDPerf$^{-/-}$ (Figure 3.7A and B). In details, mean percentage of GvHD effector cells was 11.92% (SD: 9.78%, 95% CI: 6.71-17.13) and 12.32% (SD: 8.20%, 95% CI: 8.10-16.53) in the spleen of GvHDPerf$^{-/-}$ in the absence or presence of MSC treatment, respectively. Similarly, GvHD effector cell infiltration was 27.92% (SD: 15.55, 95% CI: 9.92-35.91) and 22.17% (SD: 10.55, 95% CI: 16.55-27.79) in lungs of treated or untreated animals, respectively (Figure 3.7B).

We conclude that MSC apoptosis is indispensable for immunosuppression and requires functionally activated cytotoxic cells in the recipient.

Figure 3.7. MSC immunosuppression is abrogated in the absence of cytotoxic cells.

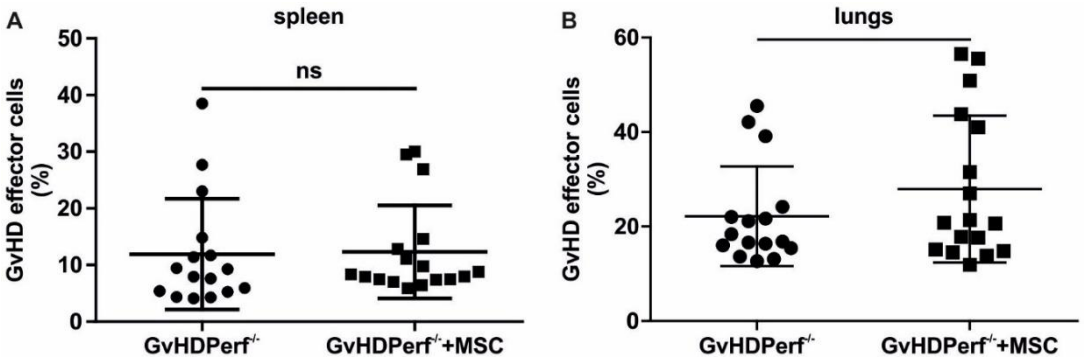

Figure 3.7. MSC immunosuppression is abrogated in the absence of cytotoxic cells. A-B: The percentage of effector GvHD cells ($CD8^+V_\beta8.3^+$) in the lymphocyte population was measured in the spleen (A) and lungs (B) of untreated $GvHDPerf^{-/-}$ (N=16) and $GvHDPerf^{-/-}$ (N=17) mice treated with MSC (mean±SD of 4 independent experiments). Statistics: unpaired t-test. ns: not significant.

3.2.3 Cytotoxic activity against MSCs is associated with clinical response to MSCs in GvHD patients.

Based on these findings, we inferred that the presence of cytotoxic cells in the recipient could be predictive of clinical responses to MSCs. We collected PBMCs from 32 patients affected by steroid-resistant aGvHD who were candidate to receive MSCs. Patient characteristics are summarized in Table 3.1. Twenty (62.5%) patients were treated in the UK, while the remaining were enrolled in Germany. Median age was 49 years (range: 3-69), 27 (87%) were males. All patients were transplanted because of a malignancy.

Fourteen patients (45%) received a transplant from a sibling, 12 (39%) from an unrelated donor, 3 (10%) from a haploidentical donor, and in 2 (6%) HSCs were obtained from cord blood. Twenty-four (77%) had a myeloablative conditioning regimen, while in the remaining 7 (23%) a reduced intensity regimen was used.

GvHD prophylaxis mostly comprised cyclosporine A (CSA) alone or in combination with methotrexate (MTX) or Mycophenolate (MMF). In 26 (81%) patients MSCs were used after at least two previous treatments, while only in 3 patients (9%) MSCs were used as second line after failure of steroids (2 patients) or CSA (1 patient).

Skin, gut, and liver were affected in 14 (43.8%), 26 (81.2%), and 6 (18.8%) of the patients. Most patients had multiorgan GvHD, while only in two (6.3%), 12 (37.5%), and 1 (3.1%) patients, skin, gut or liver were the only organs affected, respectively.

Median time from HSCT or GvHD diagnosis to MSC treatment was 92 (33-564) or 23 (4-163) days, respectively. Median dose of MSCs was $1.2x10^6$/Kg body weight (range 0.7-7.4). Response to MSC treatment was assessed as described in Material and Methods. After 33 infusions, responses were observed in thirteen cases (39.4%), in all cases a partial response was reported. When we studied the distribution of responses in the patients treated in the UK and in Germany, we could find that in the former group of patients, partial response was achieved in 11 out of 20 patients (55%), while in the latter only 2 out of 12 patients (17%) were partial responders (Figure 3.8). In all patients, MSC infusions were well tolerated.

Figure 3.8. Distribution of clinical responses amongst patients treated in the UK and Germany.

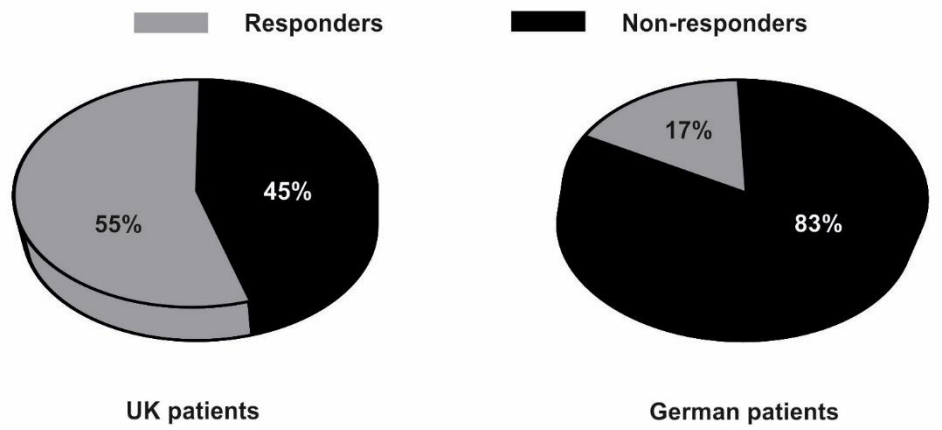

Figure 3.8. Distribution of clinical responses amongst patients treated in the UK and Germany. Distribution of responders and non-responders in patients who were treated in the UK or in Germany.

Table 3.1. Patients' characteristics.

Total (n)	31
Age, years	
Median (Range)	48yr (3-69yr)
Sex	
Male	28
Female	4
Disease*	
AML	9
ALL	3
CML	3
MDS/MPNs	5
NHL/HL/MM/Other malignancies	10
Time from HSCT to MSC treatment	
Median (range, days)	92 (33-406)
Time from GvHD to MSC treatment	
Median (range, days)	23 (4-163)
Type of transplant*	
Sibling	14
Unrelated	11
Umbilical cord	2
Haploidentical	3
Conditioning Regimen*	
Myeloablative	24
Reduced Intensity	6
GvHD prophylaxis, n	
MTX	11
CSA	15
MMF	6
Other drugs	7
GvHD treatment before MSC infusion, n**	
Methylprednisolone Alone	2
Methylprednisolone in combination with other drugs	26
Other drug combination not including Methylprednisolone	1
CSA	15
MMF	10
anti-TNFα (Infliximab, Etanercept)	12
Tacrolimus	4
MTX	1
ECP	4

anti-CD52 (Alemtuzumab)	3
ATG	1
JAK1/2 Inhibitors (Ruxolitinib)	5
Imatinib	2
Budesonide	3
GvHD grade, n	
I-II	2
III-IV	28
GvHD affected organ, n	
Skin	14
Gut	26
Liver	6

aGvHD: acute Graft versus Host disease, **AML:** Acute Myeloid Leukemia, **ALL:** Acute Lymphoblastic Leukemia, **ATG:** anti-thymocyte globulin, **CML:** Chronic Myeloid Leukemia, **CSA:** Cyclosporin, **ECP:** Extracorporeal photopheresis, , **JAK1/2:** Janus kinase 1/2, **HSCT:** Hematopoietic Stem Cell Transplant, **MEP:** Methylprednisolone, **MDS/MPNs:** Myelodisplastic Syndrome/Myeloproliferative Neoplasms, **MMF:** Mycophenolate, **MSC:** Mesenchymal Stromal Cells, **MTX:** Methotrexate, **NHL/HL/MM:** Non-Hodgkin Lymphoma/Hodgkin Lymphoma/Multiple Myeloma, **TNFα:** Tumor Necrosis Factor α.

*: Information was not reported for two patients. **: Information was not reported for three patients.

PBMCs were freshly collected within the 24 hours preceding the MSC infusion and tested directly for their ability to induce MSC apoptosis *ex vivo* in a 4-hour cytotoxic assay. One patient received two doses of MSCs, and the cytotoxic assay was performed before each dose independently. In 8 assays the MSCs were sourced from the same donor used for the infusion, while in the remaining they were obtained from a different donor. At the time of performing the assay and cytofluorimetric analysis the operator was blind to patients' clinical details. PBMCs from healthy donors (N=5) were used as controls.

Overall, PBMCs from GvHD patients exhibited a higher cytotoxicity against MSCs in comparison with that observed when PBMCs from healthy donors were used. Mean percentage of annexin $V^+/7AAD^-$ MSCs was 12,36% (SD: 12.07, 95% CI: 8.08-16.64) and 3.82% (SD: 2.50, 95% CI: 0.72-6.93) when MSCs were in co-culture with PBMCs obtained from GvHD patients or healthy controls (HC), respectively (p=0.04). However, the cytotoxicity was broadly variable, ranging from 0% to 62.3%. We then divided patients into two sub-categories according to MSC response (responders and non-responders), and analyzed the distribution of the results of the cytotoxic assay among them. We found that cytotoxicity was markedly higher when PBMCs were isolated from clinical responders in comparison with non-responders or healthy controls. Indeed, when MSCs were in co-culture with PBMCs from responding patients the mean proportion of annexin $V^+/7AAD^-$ MSCs was 19.64% (SD: 15.16, 95% CI: 10.01-29.28), three-fold higher than what observed with PBMCs from non-responders (6.90, SD: 4.75, 95% CI: 4.68-

9.13), and five-fold higher than PBMCs from healthy controls (3.82, SD: 2.50, 95% CI: 0.72-6.93) (Figure 3.9A-B).

Importantly, when we analysed the ability of our cytotoxic assay to discriminate between responders and non-responders by using the receiver-operating characteristic curve, we found that, when setting a threshold of apoptotic MSCs at 11.55%, this cut-off was predictive of clinical response with 85% sensitivity (95% CI: 54.55% to 98.08%) and 90% specificity (95% CI: 68.3% to 98.77%) (Figure 3.9C).

Figure 3.9. Cytotoxic activity against MSCs predicts clinical responses to MSCs in GvHD patients.

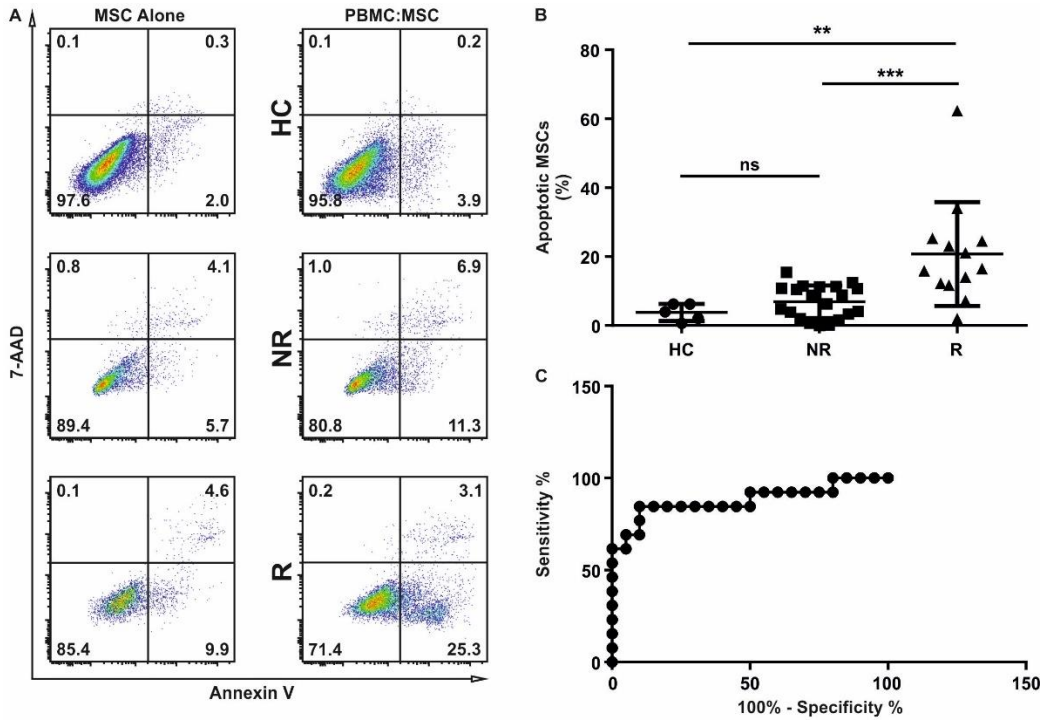

Figure 3.9. Cytotoxic activity against MSCs predicts clinical responses to MSCs in GvHD patients. A, B: PBMCs obtained from healthy controls (HC) or patients with GvHD receiving MSCs in the following 24 hours were incubated in 24-well plates with MSC at a 20:1 PBMC:MSC ratio for 4 hours. Apoptosis was measured in MSCs assessing the percentage of annexin-V^+/7-AAD^- cells by flow-cytometry. **A:** Representative plots for HC, clinical responders (R) and non-responders (NR). The panels on the left show the background apoptosis of MSC alone used in the corresponding cytotoxic assay. **B**: Apoptosis was compared among HC (circles, N=5), NR (squares; N=20), and R (triangles; N=12). Statistics: one-way ANOVA and Tukey's

Multiple Comparison test. **: p<.005, ***: p<.001 ns: not significant. **C:** Receiver-operating characteristic curve (ROC) was performed to assess specificity and sensibility of the functional assay to predict the clinical outcome of patients treated with MSCs. **HC**: Healthy Controls. **NR**: Non-Responders. **R**: Responders

We then evaluated all possible factors that could affect the response to MSC treatment. We included in our analysis also the results of the cytotoxic assay, using the cut-off of 11.55%, and dividing our patients into two subgroups: $cyto^{high}$ patients (with cytotoxicity >11.55%) and $cyto^{low}$ (with cytotoxicity <11.55%). Notably, the only factor significantly associated with response to MSCs was the cytotoxicity against MSCs (Table 3.2). Conversely, age at transplant, HSC source, conditioning regimen, time from HSCT to MSCs, time from GvHD to MSCs, treatment before MSCs, GvHD prophylaxis, MSC dose, or blood count did not have any impact on patient responses (Table 3.2).

Table 3.2. Demographic and treatment characteristics for responders and non-responders.

	Category	NR (n=19)	PR (n=13)	p-value
Age (mean (sd))		49.56 (14.03)	37.75 (23.09)	0.092
Transplant type (%)	Sibling	6 (33.3)	8 (66.7)	0.25
	Unrelated	9 (50.0)	2 (16.7)	
	Umbilical	1 (5.6)	1 (8.3)	
	Haplo	2 (11.1)	1 (8.3)	
Transplant intensity (%)	Myeloablative	14 (77.8)	10 (83.3)	1
	RIC	4 (22.2)	2 (16.7)	
MSCs to HSCT (mean (sd))		118.00 (84.65)	125.92 (135.08)	0.85
MSC to GvHD (mean (sd))		29.29 (40.72)	38.82 (34.10)	0.539
Treatment before MSC				
Methylprednisolone (%)	No	1 (5.6)	0 (0.0)	1
	Yes	17 (94.4)	11 (100.0)	
CSA (%)	No	8 (44.4)	6 (54.5)	0.71
	Yes	10 (55.6)	5 (45.5)	
MTX (%)	No	17 (94.4)	11 (100.0)	1
	Yes	1 (5.6)	0 (0.0)	
MMF (%)	No	12 (66.7)	7 (63.6)	1
	Yes	6 (33.3)	4 (36.4)	
Anti TNFa (%)	No	9 (50.0)	8 (72.7)	0.273
	Yes	9 (50.0)	3 (27.3)	
Budeboside (%)	No	16 (88.9)	10 (90.9)	1
	Yes	2 (11.1)	1 (9.1)	
ECP (%)	No	16 (88.9)	9 (81.8)	0.622
	Yes	2 (11.1)	2 (18.2)	
Imanitib (%)	No	17 (94.4)	10 (90.9)	1
	Yes	1 (5.6)	1 (9.1)	
Tacrolimus (%)	No	16 (88.9)	9 (81.8)	0.622
	Yes	2 (11.1)	2 (18.2)	
Roxolitinib (%)	No	14 (77.8)	10 (90.9)	0.622
	Yes	4 (22.2)	1 (9.1)	
ATG (%)	No	17 (94.4)	11 (100.0)	1
	Yes	1 (5.6)	0 (0.0)	
Anti CD52 (%)	No	16 (88.9)	10 (90.9)	1
	Yes	2 (11.1)	1 (9.1)	

	Category	NR (n=19)	PR (n=13)	p-value
Age (mean (sd))		49.56 (14.03)	37.75 (23.09)	0.092
Transplant type (%)	Sibling	6 (33.3)	8 (66.7)	0.25
	Unrelated	9 (50.0)	2 (16.7)	
	Umbilical	1 (5.6)	1 (8.3)	
	Haplo	2 (11.1)	1 (8.3)	
Transplant intensity (%)	Myeloablative	14 (77.8)	10 (83.3)	1
	RIC	4 (22.2)	2 (16.7)	
MSCs to HSCT (mean (sd))		118.00 (84.65)	125.92 (135.08)	0.85
MSC to GvHD (mean (sd))		29.29 (40.72)	38.82 (34.10)	0.539
Treatment before MSC				
Methylprednisolone (%)	No	1 (5.6)	0 (0.0)	1
	Yes	17 (94.4)	11 (100.0)	
CSA (%)	No	8 (44.4)	6 (54.5)	0.71
	Yes	10 (55.6)	5 (45.5)	
MTX (%)	No	17 (94.4)	11 (100.0)	1
	Yes	1 (5.6)	0 (0.0)	
MMF (%)	No	12 (66.7)	7 (63.6)	1
	Yes	6 (33.3)	4 (36.4)	
Anti TNFa (%)	No	9 (50.0)	8 (72.7)	0.273
	Yes	9 (50.0)	3 (27.3)	
Budeboside (%)	No	16 (88.9)	10 (90.9)	1
	Yes	2 (11.1)	1 (9.1)	
ECP (%)	No	16 (88.9)	9 (81.8)	0.622
	Yes	2 (11.1)	2 (18.2)	
Imanitib (%)	No	17 (94.4)	10 (90.9)	1
	Yes	1 (5.6)	1 (9.1)	
Tacrolimus (%)	No	16 (88.9)	9 (81.8)	0.622
	Yes	2 (11.1)	2 (18.2)	
Roxolitinib (%)	No	14 (77.8)	10 (90.9)	0.622
	Yes	4 (22.2)	1 (9.1)	
ATG (%)	No	17 (94.4)	11 (100.0)	1
	Yes	1 (5.6)	0 (0.0)	
Anti CD52 (%)	No	16 (88.9)	10 (90.9)	1
	Yes	2 (11.1)	1 (9.1)	

ATG: anti-thymocyte globulin, **CSA:** Cyclosporin, **ECP:** Extracorporeal photopheresis, **JAK1/2:** Janus kinase 1/2, **MEP:** Methylprednisolone, **MMF:**

Mycophenolate, **MSC:** Mesenchymal Stromal Cells, **MTX:** Methotrexate**,** **TNFα:** Tumor Necrosis Factor α.

An important question related to the cytotoxic assay is whether the results obtained could be dependent on the specific MSC donor or batch used to perform the assay. To address this important issue, we tested patients' PBMCs against the MSCs used for the infusion and compared the results of the assay with those obtained by using another MSC preparation isolated from a different and unrelated donor. In this experiment MSC1 and MSC2 were infused into patient 1 and patient 2, respectively. We found that when PBMCs obtained from patient 1 were used in our cytotoxic assay, the level of annexin $V^+/7AAD^-$ cells were similar in MSC1 (14,2%) and in MSC2 (13,2%). Similarly, when PBMCs from patient 2 were employed, the levels of apoptosis in both MSCs was almost identical (MSC1: 21.1%, MSC2: 20.9%) (Figure 3.10A). Taken together, our findings strongly support the notion that cytotoxicity did not vary amongst MSC preparations.

To further confirm the dispensable role of the specific MSC batch in the cytotoxic assay, we evaluated the susceptibility of MSCs sourced from different unrelated donors to undergo apoptosis after exposure to 4 different mixed lymphocyte reaction (MLR) combinations. We decided to use PBMCs from a MLR because it is considered an *in vitro* surrogate of alloreactivity and GvHD[384], thus closely recapitulating the use of PBMCs activated *in vivo* during GvHD.

The proportion of apoptotic MSCs was similar amongst the different MSC preparations when the same MLR was tested. This similarity was confirmed by the study of the coefficients of variation calculated using the values of MSC

apoptosis obtained with each MLR. Conversely, the cytotoxic activity against the same MSCs varied amongst different MLR (Table 3.3 and Figure 3.10B).

Table 3.3. Level of MSC apoptosis depends on specific MLR preparations.

	MLR1	MLR2	MLR3	MLR4
Mean level of MSC apoptosis	49	48.33	34.3	16.4
Std. Deviation	2.326	4.562	2.404	1.556
Std. Error of Mean	1.343	2.634	1.7	1.1
Lower 95% CI of mean	43.22	37	12.7	2.423
Upper 95% CI of mean	54.78	59.67	55.9	30.38
Coefficient of variation	4.75%	9.44%	7.01%	9.49%

Figure 3.10. Cytotoxicity against MSCs varies amongst PBMC donor.

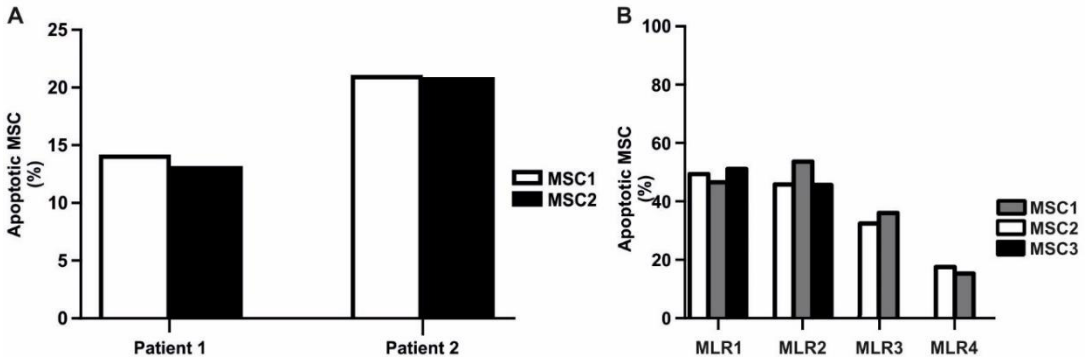

Figure 3.10. Cytotoxicity against MSCs varies amongst PBMC donor. A: PBMCs obtained from 2 different GvHD patients (Patient 1 and Patient 2) were tested for their cytotoxic activity against MSCs from two different donors (MSC1 and MSC2). MSC1 and MSC2 were infused into patient 1 and patient 2, respectively. **B:** apoptosis in MSCs obtained from different donors (MSC1, MSC2 and MSC3) after incubation with PBMCs from four different MLR responder/stimulator combinations (MLR1, MLR2, MLR3, MLR4). In A and B, the level of apoptosis was assessed by flow-cytometry after 4 hours of co-culture.

We have shown that responders and non-responders exhibited different cytotoxicity against MSCs (Figure 3.9A-B). We then studied the frequency of $CD8^+$ cells and NK cells ($CD56^+$) in both categories of patients, and we could rule out the possibility that different proportions of these cells could account for the observed difference in cytotoxic activity. Indeed, the average frequency of $CD8^+$ in the PBMCs of responders (2.83%, SD: 0.68, 95% CI: 1.99-3.68) was not different from the frequency of these cells in non-responders (3.40, SD: 3.00, 95% CI: 1.25-5.55) (Figure 3.11A). Similar results were obtained when the frequency of NK cells was considered in both category of patients. $CD56^+$ cells were 15.25% (SD: 11.1, 95% CI: 1.47-29.03) in responders, and 11.33% (SD: 8.83, 95% CI: 5.01-17.64) in non-responders (Figure 3.11B). Therefore, we conclude that the presence of activated cytotoxic cells in the recipient is predictive of MSC therapeutic activity.

Figure 3.11. Cytotoxicity against MSCs is independent of the percentage of CD8$^+$ or CD56$^+$ in GvHD patients.

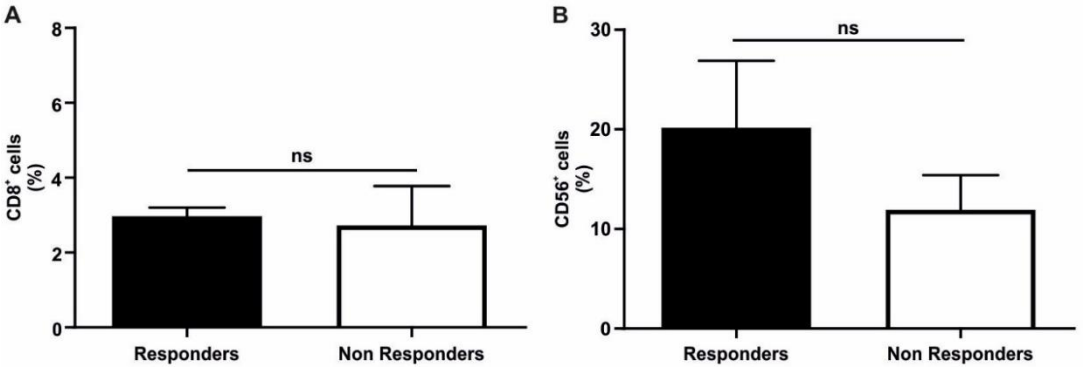

Figure 3.11. Cytotoxicity against MSCs is independent of the percentage of CD8$^+$ or CD56$^+$ in GvHD patients. A, B: PBMCs obtained from 11 GvHD patients (R: 3, NR: 8) were analysed for the percentage of CD8$^+$ (**A**) and CD56$^+$ (**B**) cells. Statistics: unpaired t-test. ns: not significant. **NR**: Non-Responders. **R**: Responders

3.2.4 MSC apoptosis induced by cytotoxic cells is the result of a bystander effect.

To define the mechanisms that drive apoptosis in MSCs, we used *in-vitro*-activated PBMCs from healthy donors as effector cells. PBMCs were pre-activated incubating them with PHA (5 µg/ml) for 72 hours, or in a MLR for 5 days. MSCs were then plated in 24 well plate alone, or in co-culture with PHA or MLR pre-activated PBMCs at different PBMC:MSC ratios (2.5/1 to 40/1). The level of apoptosis within MSCs was then assessed by Annexin V/7-AAD staining using flow-cytometry at different time points (30 minutes to 24 hours). We found that activated but not resting PBMCs induced extensive early apoptosis (annexin-V^+/$7AAD^-$) in MSCs (Figure 3.12A). This apoptosis peaked at 4 hours reaching a plateau at a PBMC:MSC ratio 20/1 (mean 84.27%, SD: 3.3, 95% CI: 76.15-92.38) and shifted towards late apoptosis (annexin-V^+/$7AAD^+$) by 24 hours (Figure 3.12B). When PBMCs activated with MLR were used, a plateau was reached at a PBMC:MSC ratio 20/1, and the level of apoptosis in MSCs was slightly lower (mean 61.75%, SD: 3.89, 95%: 26.81-96.69).

Figure 3.12. MSC apoptosis is induced within the first 4 hours when in contact with activated PBMCs.

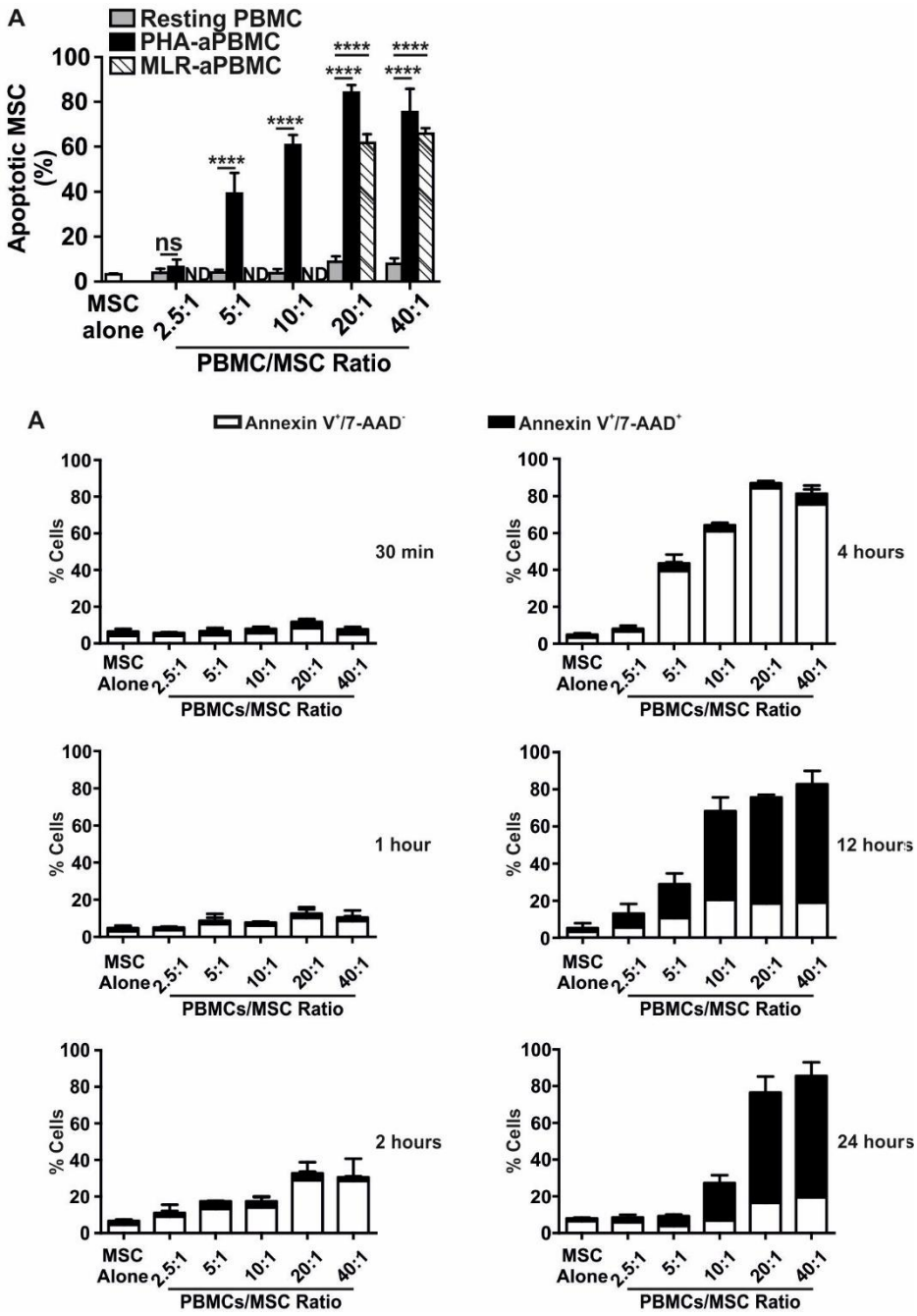

Figure 3.12. MSC apoptosis is induced within the first 4 hours when in contact with activated PBMCs. A: PBMCs from healthy donors (each

independent experiment used a different PBMC donor) were activated using PHA (PHA-aPBMC) or MLR (MLR-aPBMC). Resting (grey bars), PHA-aPBMCs (black bars) or MLR-aPBMCs (dashed bars) were incubated with MSCs at the indicated ratios and MSC apoptosis (annexin-V^+/7-AAD^-) calculated after 4 hours. ND: Not done. **B:** PHA-aPBMCs were incubated with MSCs at escalating PBMC:MSC ratios. MSC apoptosis was assessed by annexin-V/7-AAD at different time-points by flow-cytometry. Results represent the mean±SD of 3 independent experiments with PHA-aPBMCs and 2 independent experiments when MLR-aPBMCs were employed. Statistics: one-way ANOVA, with Tukey's Multiple Comparison Test. ****: $p<.0001$. ns: not significant.

In order to confirm that annexin V expression in MSCs exposed to activated PBMCs was the result of caspase activation, MSCs were transfected with the pECFP-DEVDR-Venus vector. EC-RP construct is composed of a FRET donor-acceptor pair ECFP and venus (YFP) connected via a flexible linker containing the caspase cleavage sequence $DEVDR^{380}$. In this system, in the absence of caspase 3 activity, the linker is intact, and energy can be transferred between the two fluorochromes. Such a condition translates in reduction of the signal of the donor fluorochrome (ECFP) and increase of that of the acceptor (YFP). Conversely, when the linker is cleaved in the presence of caspase 3 activation, energy transfer is lost, and ECFP and YFP signals result increased and reduced, respectively. Our data demonstrated that caspase 3 is indeed activated in MSCs and, in accord with our *in vivo* observations (Figure 3.2A and B), this activation could be induced only by activated PBMCs with a peak at 90 minutes (Video 3.1, 3.2 and 3.3).

The crucial role played by caspase 3 was confirmed by the observation that MSC apoptosis was completely abrogated by the pan-caspase inhibitor Z-VAD-FMK (Figure 3.13A and B, and Video 3.4). Indeed, annexin V^+/7-AAD^- MSCs were 46.20% (SD: 4.68, 95% CI: 35.57-58.83), 42.97% (SD: 9.05, 95% CI: 20.46-65.47) and 7.09% (SD: 0.12, 95% CI: 6.78-7.39) when MSCs were in co-culture with activated PBMCs, activated PBMCs and vehicle, or activated PBMCs and Z-VAD-FMK, respectively (Figure 3.13A). As expected, these data were confirmed when caspase 3 activation was evaluated using pECFP-DEVDR-transfected MSCs in the presence of activated PBMCs and Z-VAD-FMK (Figure 3.13B).

Figure 3.13. MSC apoptosis is caspase 3 dependent.

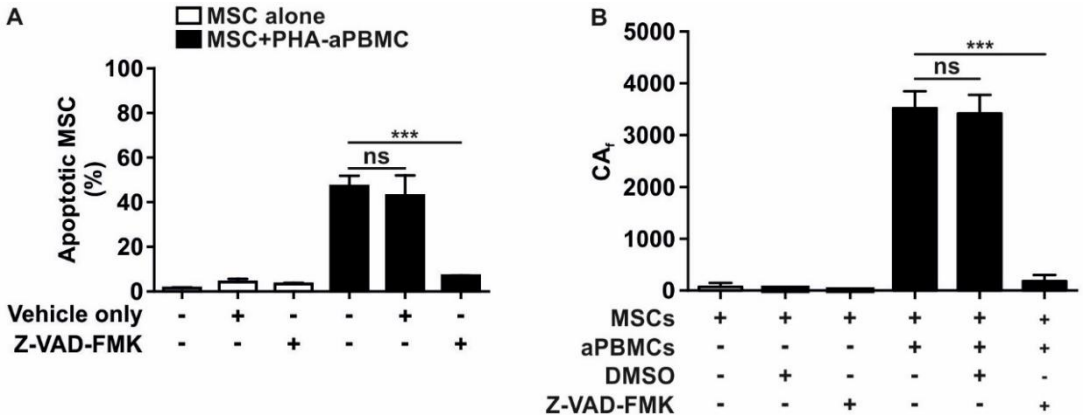

Figure 3.13. MSC apoptosis is caspase 3 dependent. A: Apoptosis in MSC cultivated with MLR-aPBMC at a PBMC:MSC ratio 20:1 in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK (10 µM) or the corresponding concentration of its vehicle (DMSO). **B:** MSCs were transfected with the pECFP-DEVDR-Venus vector (FRET-MSC) and FRET between pECFP and Venus-YFP FRET was studied by flow-cytometry and Caspase activity (CAf) calculated as described in Materials and Methods[380]. FRET-MSCs were cultured alone, with PHA-aPBMCs, or PHA-aPBMCs in the presence of Z-VAD-FMK (50 µM). The PHA-aPBMC:MSC ratio was 40/1. Results represents the mean±SD of 3 independent experiments. Statistics: one-way ANOVA, with Tukey's Multiple Comparison Test. ***: p<.001. ns: not significant.

In order to identify the cells inducing apoptosis in MSCs, we performed selective enrichment and depletion experiments amongst activated PBMCs. $CD56^+$ or $CD8^+$ cells positively selected from MLR-aPBMCs were able to induce apoptosis in 38.9% (SD: 18.5, 95% CI: 23.41-54.39) and 21.6% (SD: 3.9, 95% CI: 15.33-27.87) of MSCs in co-culture, levels which were similar to those obtained when unfractionated MLR-aPBMCs were used (35.9%, SD: 14.7, 95% CI: 20.49-51.31). Conversely, when $CD11b^+$ or $CD4^+$ cells were selected, significantly reduced levels of MSC apoptosis was observed (Figure 13.14A). Importantly, only when both $CD8^+$ and $CD56^+$ cells were depleted from MLR-aPBMCs, the induction of MSC apoptosis could be significantly reduced. Annexin V^+/7-AAD^- MSCs were 12.0% (SD: 4.54, 95% CI: 0.73-23.29) when in co-culture with MLR-aPBMCs depleted of $CD8^+$/$CD56^+$, in contrast, when unfractionated PBMC were used, MSC apoptosis was 35.9% (SD: 14.68, 95% CI: 20.49-51.31) (Figure 3.14B). Taken together, these results demonstrate that both $CD56^+$ natural killer (NK) and $CD8^+$ T cell populations were the only cells responsible for initiating MSC apoptosis.

Figure 3.14. Activated CD56$^+$ and CD8$^+$ cells are the only populations able to induce MSC apoptosis.

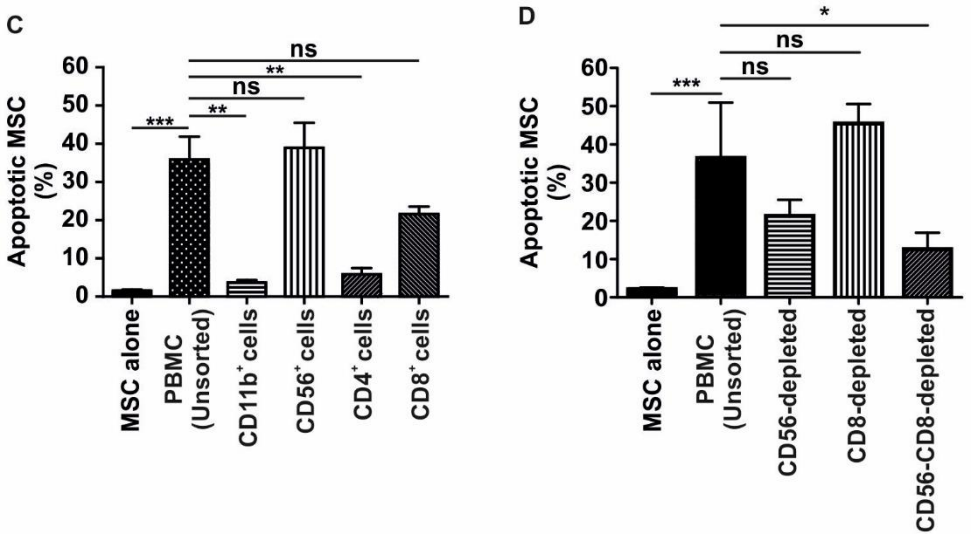

Figure 3.14. Activated CD56$^+$ and CD8$^+$ cells are the only populations able to induce MSC apoptosis. **A-B:** Apoptosis in MSCs cultivated with MLR-aPBMC used as unfractionated or positively selected for CD11b$^+$, CD4$^+$, CD8$^+$ or CD56$^+$ cells (**A**) or depleted of CD56$^+$, CD8$^+$ or both (**B**). In all experimental conditions, PBMC:MSC ratio was 20/1. Results represent the mean±SD of 3 or independent experiments. Statistics: one-way ANOVA, with Tukey's Multiple Comparison Test. *: p<0.5. **: p<.01. ***: p<.001. ns: not significant.

To characterize the mechanisms mediating MSC apoptosis induced by activated cytotoxic cells, we studied potential factors involved in caspase 3 activation and mediated by cytotoxic cells through the immunological synapse. We specifically assessed the role of GrB and perforin[385,386], HLA-I, HLA-II complexes[387], the adhesion molecule CD44[388], the CXCL12/CXCR4 axis[389], the very late antigen-4 (VLA-4) ($\alpha_4\beta_1$)/VCAM complex[390], ICAM complex[387], Integrins such as $\alpha_V\beta_3$ and CD18[391], and the microtubule organizing center[392]. Furthermore, we assessed the role of CD95 (FAS), TNFα and TRAIL, since their importance in mediating extrinsic receptor-mediated apoptosis[393].

The inhibition of either GrB or perforin completely abolished the ability of activated PBMCs to induce MSCs apoptosis (Figure 3.15A) and activate caspase 3 (Figure 3.15B, Video 3.5 and 3.6). In the presence of GrB and perforin inhibitors, annexin V^+/7-AAD^- MSCs were 7.8% (SD: 1.81, 95% CI: 3.30-12.33) and 8.9% (SD: 4.83, 95% CI: -3.03-20.96), respectively, thus significantly reduced in comparison with the percentage of apoptotic MSCs obtained in the absence of any inhibition (43.43%, SD: 14.7, 95% CI: 6.90-79.96) (Figure 3.15A). Similarly, caspase 3 activity, calculated as described by He and colleagues[380], was 3519 (SD: 323.7, 95% CI: 2715-4323) in the positive control culture (MSC/MLR-activated PBMCs without inhibitors), while it was 102.6 (SD: 3.54, 95% CI: 70.73-134.4) and 845.2 (SD: 731.3, 95% CI: -5726-7416) in the presence of GrB or perforin inhibitors, respectively (Figure 3.15B). We also observed reduced PBMC-mediated cytotoxicity when CD95 ligand (CD95L, also known as FasL or APO-1L) was neutralized (Figure

3.15C). In contrast, MSC apoptosis was not reduced when TNFα or TRAIL were inhibited, even in the presence of very high concentrations of their respective inhibitors (Figure 3.15D).

We then interrogated the nature of the MSC-cytotoxic cell interaction. We observed that apoptosis was not affected by the presence of anti-HLA class I- or anti-HLA class II neutralizing antibodies. Consistently, the cytotoxic activity of activated PBMCs against autologous or allogeneic MSCs did not differ (Figure 3.15E). However, although PBMCs required physical contact with MSCs to induce apoptosis (Figure 3.15F), blocking immunological synapse formation by inhibiting the polarization of microtubule organizing center[392] had no effect (Figure 3.15G). Similar results were obtained when the adhesion molecule CD44, the CXCL12/CXCR4 axis, the VLA-4 ($\alpha_4\beta_1$)/VCAM complex, ICAM complex, or integrins such as $\alpha_V\beta_3$ and CD18, were neutralized by specific antibodies or antagonists. These results demonstrate that MSC killing by activated cytotoxic cells is a bystander effect that does not involve the immunological synapse.

Figure 3.15. MSC apoptosis is mediated by Gr B/perforin and FAS/FAS-L and is the result of a bystander effect.

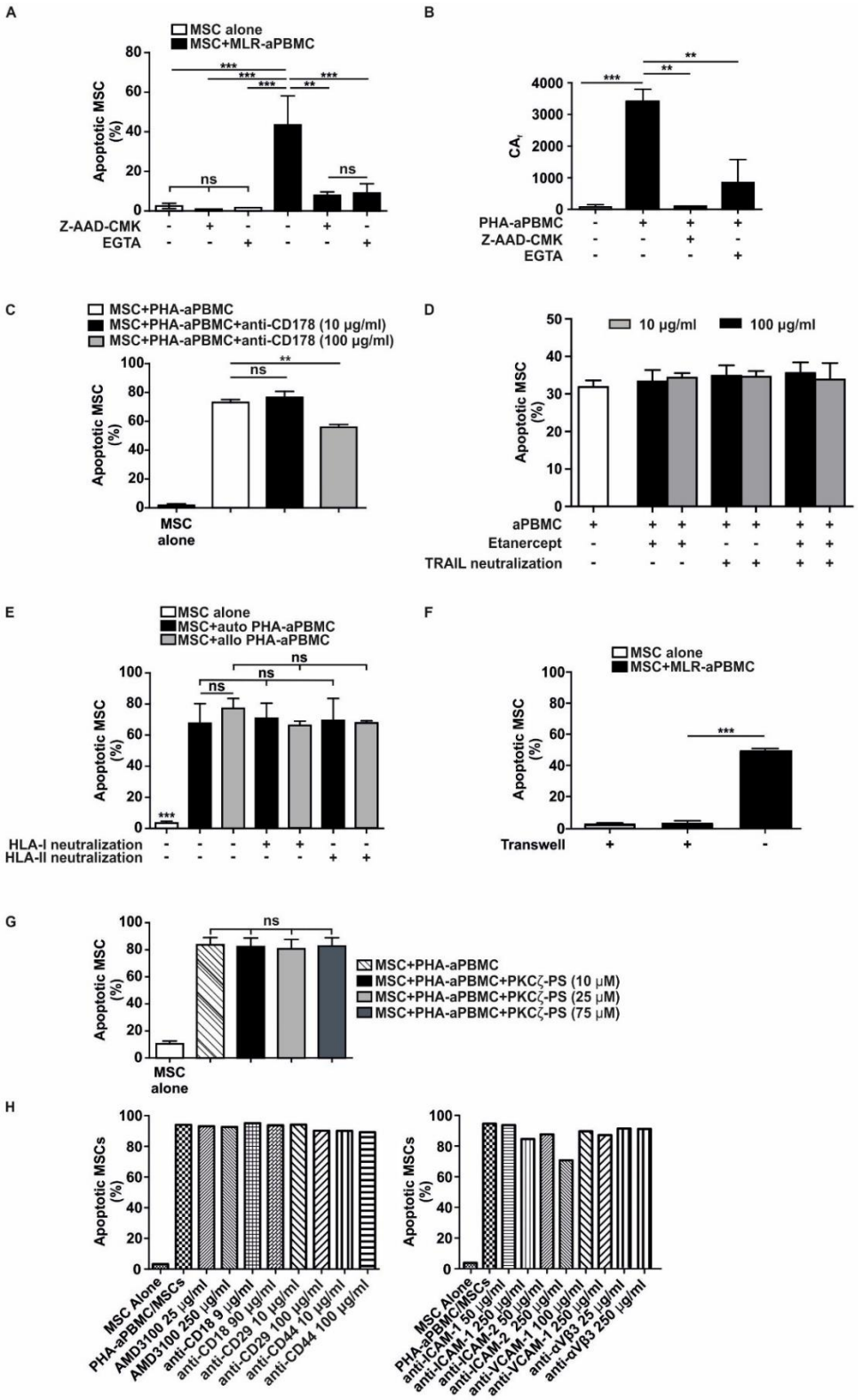

Figure 3.15. MSC apoptosis is mediated by Gr B/perforin and FAS/FAS-L and is the result of a bystander effect. A: Apoptosis in MSCs cultivated with MLR-aPBMC in the presence or absence of the GrB inhibitor Z-AAD-CMK (300 µM) or the perforin inhibitor ethylene glycol-bis(2aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (4 mM). **B:** MSCs were transfected with the pECFP-DEVDR-Venus vector (FRET-MSC) and FRET between pECFP and Venus-YFP FRET was studied by flow-cytometry and Caspase activity (CAf) calculated as described in Materials and Methods[380]. FRET-MSCs were cultured alone, with PHA-aPBMCs, or PHA-aPBMCs (at a PBMC:MSC ratio 40:1) in the presence of GrB inhibitor or the perforin inhibitor as in A. **C:** Apoptosis in MSCs cultivated with PHA-aPBMCs in the presence or absence of neutralizing concentrations (10 µg/ml and 100 µg/ml) of FAS-L mAb anti-CD178. **D:** MLR-aPBMCs were cultivated with MSCs. Where indicated, the TNF-α inhibitor Etanercept or the mAb anti-TRAIL were used at 10 µg/ml or 100 µg/ml. **E:** Apoptosis in MSCs after culture with autologous (black bars) or allogeneic (grey bars) PHA-aPBMCs in the presence or absence of neutralizing doses of anti-HLA-A-B-C or anti-HLA-DR antibodies. The white bar shows spontaneous apoptosis in MSCs plated alone. **F:** Apoptosis in MSCs cultivated with MLR-aPBMCs in direct contact or in a transwell. **G:** Apoptosis in MSCs cultivated with PHA-aPBMCs in the presence or absence of escalating doses (10 to 75 µM) of PKCζ-PS. **H:** Apoptosis in MSCs cultivated with PHA-aPBMCs in the presence or absence of escalating doses of AMD3100 (antagonist of CXCR4, for blockage of the CXCL12/CXCR4 axis), and of neutralizing antibodies against Integrin CD18 and $\alpha_V\beta_3$, CD29

(which forms with CD49d VLA-4), the adhesion molecule CD44, ICAM1, ICAM2 and VCAM1. In A, C-H the PBMC:MSC ratio was 20:1. In all settings, MSC apoptosis was evaluated by flow-cytometry after 4 hours of co-incubation. Results represent the mean±SD of 3 (A-E, and G) or 6 (F) independent experiments. Statistics: one-way ANOVA, with Tukey's Multiple Comparison Test. **: p<.01. ***: p<.001. ns: not significant.

3.2.5 MSC apoptosis does not interfere with the recognition of the specific target of cytotoxic cells.

Having determined that the MSC apoptosis induced by cytotoxic cells is MHC-independent and not antigen-specific, we asked whether MSCs could exert their immunosuppressive effects by competing with and antagonizing antigen-specific recognition. NY-ESO1-specific $CD8^+$ T cell clone (4D8) or IL2-activated polyclonal $CD56^+$ purified NK cells were used as effector cells against NY-ESO-1 peptide (SLLMWITQC)-pulsed T2 or K562 cells, respectively. Two different sets of experiments were performed. In the first set, 4D8 or NK cells were tested against fixed numbers of putative (susceptible) target cells in the presence of escalating numbers of MSCs used as a cold target. The alternative condition consisted of escalating the numbers of the putative target cells – now used as cold targets – in the presence of a fixed number of MSCs then considered as the susceptible target. MSCs did not compete with antigen-specific T cell cytotoxicity since the killing of peptide-pulsed T2 cells was not affected by the presence of MSCs (Figure 3.16A). The same results were obtained using NK cells (Figure 3.16B). In contrast, the presence of the putative target cells markedly reduced MSC killing in a dose dependent manner in both systems (Figure 3.16C and D). Our data show that MSC killing does not interfere with the primary recognition of the cognate antigen.

Figure 3.16. MSCs do not compete with cytotoxic cell recognition of the cognate target.

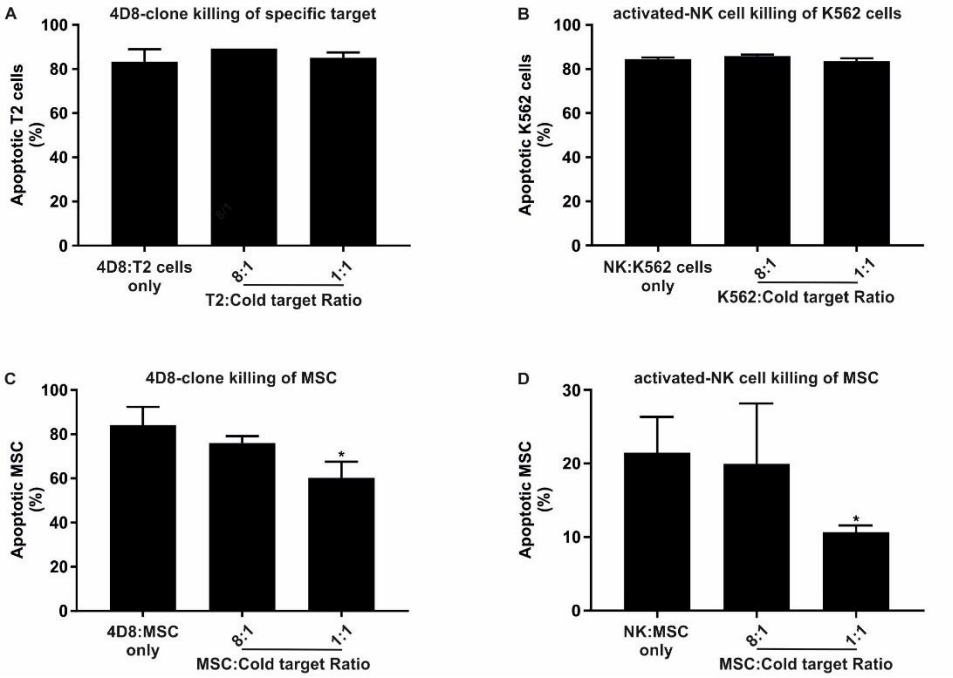

Figure 3.16. MSCs do not compete with cytotoxic cell recognition of the cognate target. A: Apoptosis in T2-cell after culture with 4D8 cells at a 20:1 4D8:T2 ratio. Where indicated increasing concentrations of MSC (used as cold target) were added. Apoptotic T2 cells were identified as annexin-V^+/7-AAD^+ cells. **B:** Apoptosis in K562 cultured with NK cells (20:1 NK:K562 ratio). Where indicated increasing concentrations of MSC (used a cold target) were added. **C:** Apoptosis in MSC cultured with 4D8 cells (20:1 4D8:MSC ratio). Where indicated increasing concentrations of T2 cells (used as cold target) were added. **D:** Apoptosis in MSC cultured with NK cells at a 20:1 NK:MSC ratio. Where indicated, increasing dilutions of K562 (used as cold target) were

added. In all experiments, apoptosis of MSCs, T2 or K562 cells was assessed after 4 hours of co-culture by flow cytometry. Results represent the mean±SD of 3 independent experiments. Statistics in A, B, C and D: one-way ANOVA and Tukey's Multiple comparison test. *: $p<.05$.

3.2.6 MSCs are not immunosuppressive in the absence of cytotoxic cells in a Th2-type inflammation model.

Our data imply that, since MSC killing does not interfere with the primary recognition of the cognate antigen, induction of apoptosis must be prominently involved in the immunosuppressive activity. Accordingly, in the GvHD model described above, MSC apoptosis produced by recipient cytotoxic cells is required for immunosuppression. Therefore, we asked whether this causative relationship remains valid in a different disease model associated with non-cytotoxic Th2-type inflammation.

We selected the model of OVA-induced allergic airway inflammation[376] summarized in Figure 20A. Although cytotoxic immune cells have been implicated as contributing to the induction of this condition[394,395], $CD8^+$ and $NK1.1^+$ cells infiltrating BAL and lung tissues were less than 2% one hour after the last OVA challenge, when MSCs were infused (Figure 3.17B-E).

Figure 3.17. Mouse model of Th2-type inflammation model: absence of cytotoxic cells in lungs and bronchoalveolar lavage.

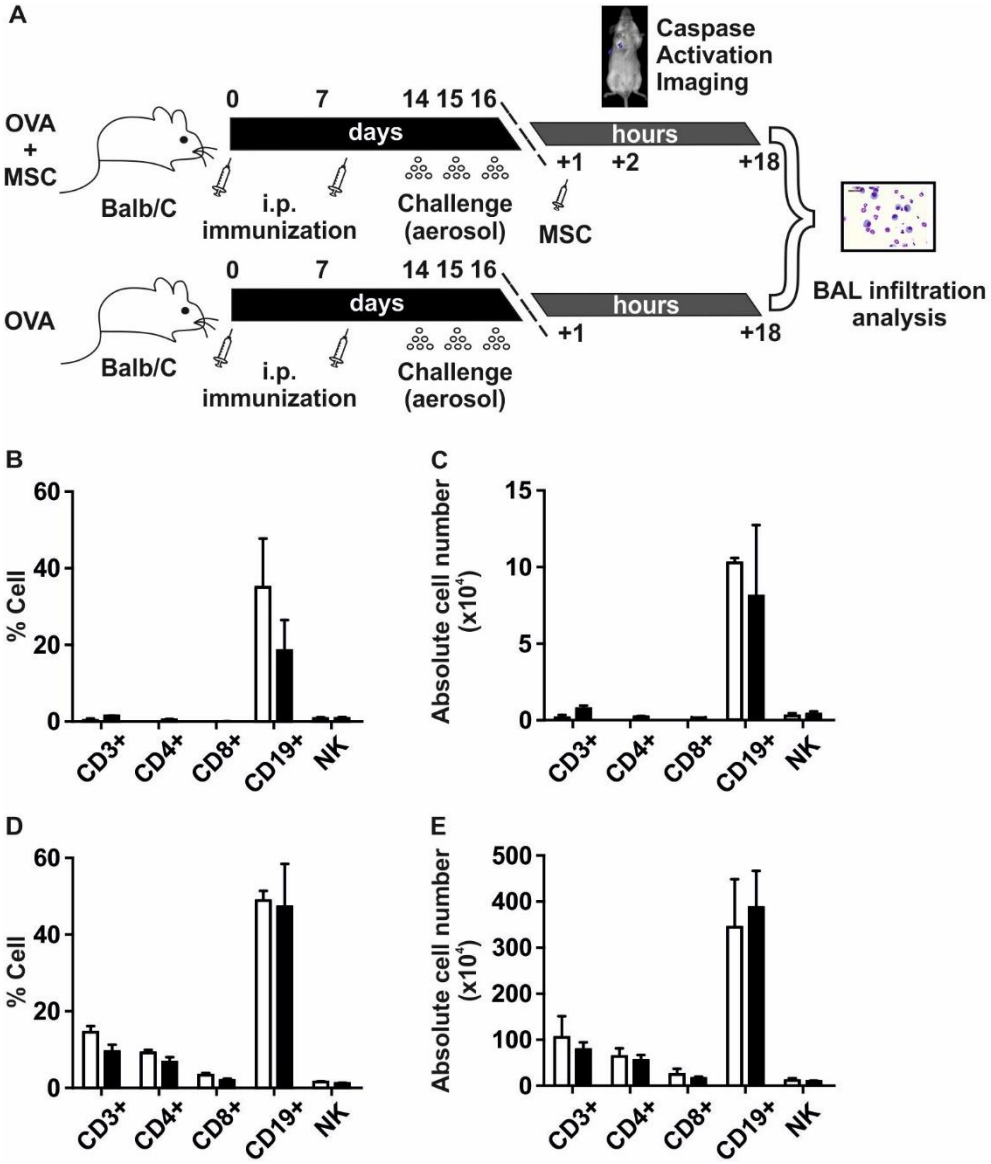

Figure 3.17. Mouse model of Th2-type inflammation model: absence of cytotoxic cells in lungs and BAL. A: Balb/C mice were immunized i.p. with OVA at day 0 and 7 and subsequently challenged with OVA through aerosol at days 14, 15 and 16 (OVA group). Experimental group was treated with

MSCs one hour after the last challenge (OVA+MSC). When luc-MSCs were used, mice were imaged one hour after infusion for the analysis of caspase 3 activation after i.p. injection of DEVD-aminoluciferin. After 18 hours from treatment, eosinophil infiltration in BAL was evaluated. **B-E:** Percentage (**B, D**) and absolute numbers (**C, E**) of different cellular types in the BAL (**B, C**) and lungs (**D, E**) of naïve (with bars) (N=3) and OVA-sensitized (black bars) (N=3) mice. Results represent the mean±SD of 3 independent experiments. In OVA-sensitized mice, the analysis was performed 1 hour after the last aerosol challenge.

To confirm the absence of MSC killing, mice received luc-MSCs to assess caspase activation after infusion and imaged one hour later. No caspase activation was detected in any of the mice (Figure 3.18A and B). To confirm that luc-MSCs can be tracked in the lungs of all animals, we subsequently infused mice with the control D-luciferin. We could find that high signal could be detected from all animals (Figure 3.18C), with not significant difference among groups. Mean TLS were 120.7 (SD: 63.62, 95% CI: 53.95-187.5), and 125.4 (SD: 36.11, 95% CI: 35.7-215.1) in OVA+MSCs and naïve mice, respectively (Figure 3.18D).

The therapeutic activity assessed by quantitating the eosinophil infiltration in the BAL showed no difference between MSC-treated and untreated mice (Figure 3.18E). Together, these results indicate that also in this model MSC immunosuppression relies on the presence of recipient cytotoxic cells that mediate MSC apoptosis.

Figure 3.18. MSCs do not have immunosuppressive activity *in vivo* in the absence of induced apoptosis in a Th2-type inflammation model.

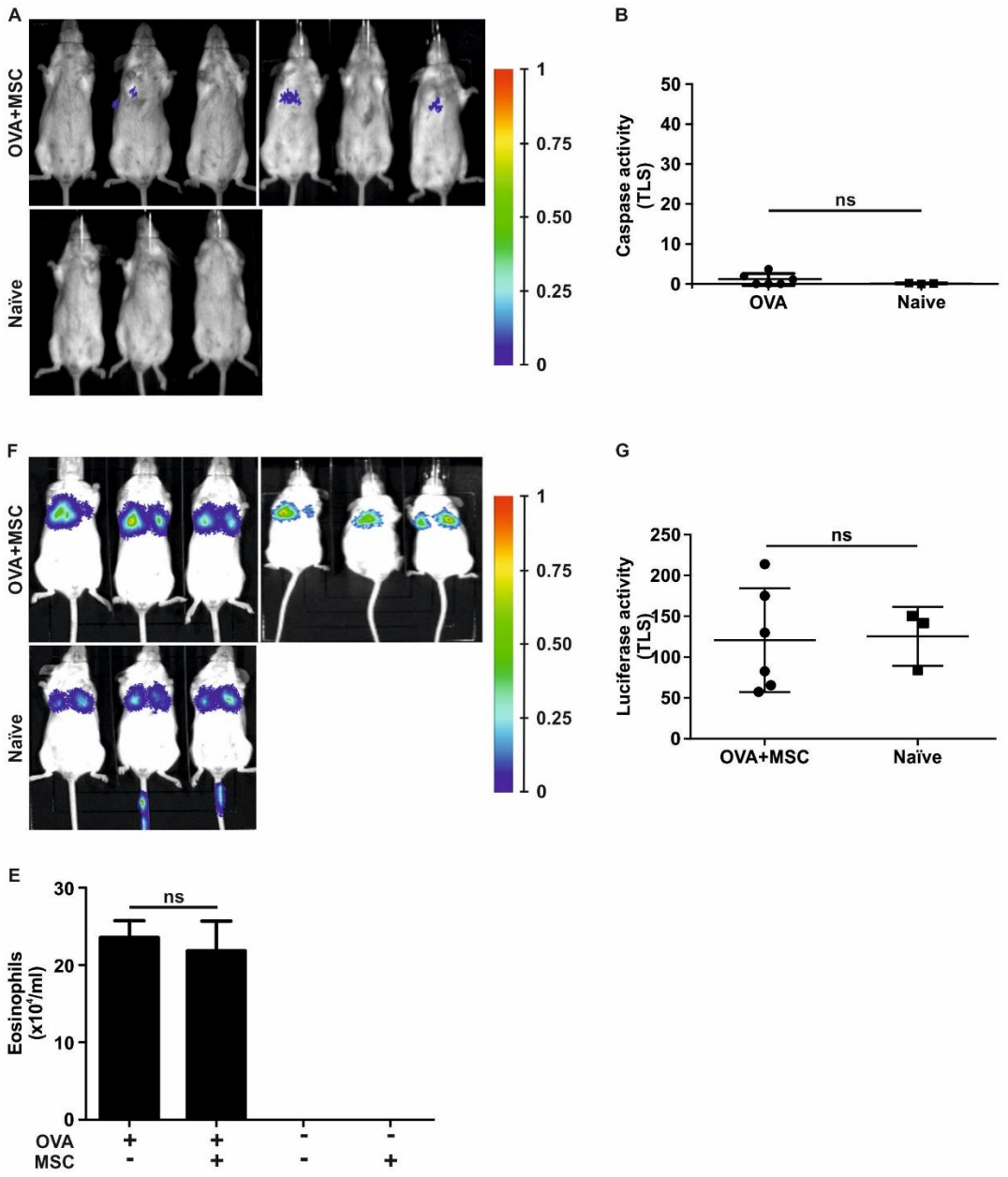

Figure 3.18. MSCs do not have immunosuppressive activity *in vivo* in the absence of induced apoptosis in a Th2-type inflammation model. A: luc-MSCs were injected into naïve (N=3) and OVA+MSCs (N=6) mice one

hour after the last challenge. One hour later, mice received DEVD-aminoluciferin and were imaged in 3 independent experiments. White lines separate multiple photographs assembled in the final image. **B:** TLS was measured from Fig. 6A (mean±SD). **C:** In order to confirm the presence of luc-MSCs in the lungs of all groups of mice infused with MSCs, the same mice imaged in A were injected with D-Luciferin. White lines separate multiple photographs assembled in the final image. **D:** TLS was measured from the images of mice in Fig. S6F and shown as mean±SD. Statistics: unpaired t-test. ns: not significant. **E:** Eighteen hours after MSC infusion, eosinophil infiltration was assessed in the BAL of naïve (N=3), naïve infused with MSCs (N=3), OVA (N=6) and OVA+MSCs (N=6) mice in two independent experiments and mean±SD are shown.

3.3 Discussion.

This study sheds light on the controversial topic of MSC therapeutics by identifying a crucial mechanism that potentially explains several unresolved issues in the field. The first striking piece of information provides the resolution to the paradox that MSCs are therapeutically efficacious despite the lack of engraftment[396–398].

We have demonstrated that MSCs undergo extensive caspase activation and apoptosis after infusion in the presence of cytotoxic cells, and that this is a requirement for their immunosuppressive function.

Our results are in line with previous studies, whereby activated but not resting NK cells were able to lyse MSCs *in vitro*[46], or MSCs were cleared *in vivo* by deployment of different recipient-dependent reactions[397–401]. Along with these observations, our data challenge the notion of MSCs as immunopriviledged. This assumption was based on the acknowledged property of MSCs to express low or no levels of HLA-I, HLA-II or other co-stimulatory molecules (such as CD40, CD80 or CD86)[1], and was supported by the MSC ability to immunosuppress rather than fail to present antigens *in vitro*[44,402,403], and to be therapeutic effective across the MHC barrier both in animal models[404] and in humans *in vivo*[26,329]. Notably, the results of our study are the first showing the instrumental role of *in vivo* MSC apoptosis in delivering immunosuppression after infusion, thus reconciling the role of the

demonstrated MSC rejection[405] in the context of their immunosuppressive functions across MHC barrier[26,329,404].

Furthermore, although several studies[375,406–408] have reported the ability of apoptotic cells to modulate immune responses, here we provide evidence that *in vivo* naturally occurring cell death drives immunosuppression.

MSC apoptosis requires and is induced by cytotoxic granules contained in recipient cytotoxic cells that also mediate GvHD in recipient mice. Importantly, the cytotoxic activity against MSCs can also be detected in the PBMCs of GvHD patients and this is predictive of clinical responses. Recently, analysis of plasma markers in patients serially monitored before and after MSC treatment has highlighted the role of a panel of biomarkers (including regenerating islet-derived 3-α, TNF receptor 1, IL-2 receptor alpha, soluble cytokeratin 18 and elafin) as predictors of GVHD-related mortality[297]. Accordingly, most of them were selectively reduced in patients responding to MSCs[10,11]. However, the role of these markers seems to be confined to the early assessment of response after treatment, rather than their use as response predictors. In contrast, our data show that patients displaying high cytotoxicity respond to MSCs, whilst those with low or absent cytotoxic activity do not improve following MSC infusion. Therefore, the ability of the recipient to generate apoptotic MSCs appears to be a requirement for the therapeutic efficacy and could be used as a potential biomarker to stratify patients for MSC infusions before the treatment. Importantly, among the several

parameters evaluated, only the results of the cytotoxic assay were significantly predictive of the response to MSCs. This was not surprising, considering the high specificity and sensibility of the test. However, the limited number of patients analyzed warrants further validation in a prospective clinical study. Moreover, characterizing the phenotype of the cytotoxic cells mediating MSC apoptosis in patients will enable the development of a more approachable assay for use in a routine pathology laboratory.

One of the impacts of our study is that, although MSCs remain the necessary starting point for therapeutic immunosuppression, patient-derived cells play a crucial role in delivering such an immunosuppression. This new perspective, in line with clinical data whereby MSCs from the same donor can give different responses in different patients[327–331,338,339], may significantly affect the Research and Development sector of MSC manufacturing. In the last decades, many efforts have been spent for the identification of the most clinically effective MSC preparations. Several strategies have been proposed, including the selection of MSCs based on biological parameters such as the magnitude of IDO synthesis[83] or the intracellular levels of the transcription factor TWIST1[410]. Conversely, other groups suggested to overcome the intrinsic variability among MSC batches by using MSCs sourced from different donors and pooled together during the expansion phase[411]. Our results seem to suggest that MSCs exhibit similar capacity to undergo apoptosis, despite different batches were used (Figure 3.10). However, the cells used in this study were all sourced from BM and expanded using the same protocol. Further studies are needed to verify whether MSCs from different sources,

administered after thawing or from fresh cultures, expanded in selected conditions, or differentially sorted based on specific features have different “capacity” to be killed. In this perspective, the cytotoxic assay may be devised as a tool for standardization of MSC manufacturing by select specific thresholds of killing used as product specification. Such an assay would also address the unmet need for a potency assay as a guideline for regulatory Authority requirements[412] to implement quality control of manufactured MSCs.

This perspective would be a complete upturn of the concept of potency assay in the MSC therapeutics. Most potency assays are designed with the aim to identify or select the “most immunosuppressive” MSC batches[413,414], but they are exclusively based on MSC *in vitro* properties. By measuring their susceptibility to undergo apoptosis when exposed to cytotoxic cells, our assay would identify the most ‘fit MSCs’, at least if administered to patients able to induce their apoptosis.

MSC recognition by cytotoxic cells is not antigen specific as neither requires HLA engagement, nor results from an alloreactive rejection, thus supporting the current practice of using third-party MSCs. MSCs must be in physical contact with the activated cytotoxic cells to undergo apoptosis, although immunological synapse is not required. This supports a bystander role for the cytotoxic granules released by the activated cytotoxic cell. Accordingly, it has been described that lytic granule secretion precedes the formation of

cytotoxic T lymphocyte/target cell synapse[392]. Furthermore, such a non-specific mechanism can mediate tissue damage in the context of human immunodeficiency virus replication[415] or atherosclerosis[416] whereby activated $CD4^+$ or NK T cells have been implicated in the progression of human immunodeficiency virus infection or the atherosclerotic disease, respectively. In these studies, bystander cells are not of mesenchymal origin, thus raising the interesting question, that we have not addressed here, of whether non-specific induction of apoptosis and subsequent immunosuppression is exclusive to MSCs.

4 Apoptotic MSCs are immunosuppressive and induce IDO production in recipient phagocytes.

4.1 Introduction

In the previous Chapter we have demonstrated that when MSCs are infused into a recipient harbouring activated cytotoxic cells they are induced to undergo apoptosis and that this apoptosis is necessary to deliver immunosuppression. Furthermore, the cytotoxic activity of GvHD patients could be used as a biomarker to predict clinical outcome.

Although these results represent important steps forward in the improvement of MSC therapeutics, they do not explain how apoptotic MSCs exert their tolerogenic properties *in vivo*. Furthermore, the results imply that a significant proportion of GvHD patients cannot benefit from this treatment, because they do not induce MSC apoptosis.

It is well-known that clearance of apoptotic cells in the steady-state is mostly an immunological silent event[417]. A crucial role in controlling the potential inflammation is played by phagocytic cells which clear apoptotic cells by engulfing them. Following this phenomenon, called efferocytosis, phagocytes

acquire a tolerogenic phenotype that exert potent immunosuppression[374,418]. By mimicking this process, several groups have used apoptotic cell-based approaches to treat pre-clinical models of immune mediated diseases[375], prolong survival of transplanted organs[419], or prevent GvHD[420]. Based on these assumptions, we tested the hypothesis that MSCs made apoptotic *in vitro* could exert *in vivo* therapeutic activity also in the absence of cytotoxic cells in the recipient and studied the underlying mechanisms.

4.2 Results

4.2.1 Apoptotic MSCs are immunosuppressive in a Th2-type inflammation model.

First, we decided to test whether *in vitro* generated apoptotic MSC (ApoMSCs) could bypass the need of cytotoxic cells and ameliorate eosinophil infiltration. Based on the observation that MSC apoptosis was induced *in vitro* by activated PBMCs through the release of GrB/perforin, along with involvement of the FAS/FAS-L pathway signaling (Figures 3.15A-C), we optimized the generation of apoptotic MSCs *in vitro* (ApoMSCs) by using recombinant GrB and a monoclonal antibody activating human FAS (Figure 4.1B and C).

When ApoMSCs were administered to recipient mice we observed that the eosinophil infiltrate in BAL was much reduced, even at extremely low doses. Indeed, eosinophil infiltration in BAL was 21.45x104/ml (DS: 7.57, 95% CI: 13.49-29.40) in OVA only mice (n=6), 8.28x104/ml (DS: 7.29, 95% CI: 1.53-15.03) in OVA mice treated with $1x10^6$ ApoMSCs (n=7), 9.01x104/ml (DS: 3.67, 95% CI: 6.18-11.84) in mice treated with $2x10^5$ ApoMSCs (n=9) and 5.2x104/ml (DS: 4.24, 95% CI: 0.74-9.65) in mice treated with $4x10^4$ ApoMSCs (n=6). No eosinophil infiltration could be found in naïve Balb/C mice receiving $1x10^6$ or 2x105 ApoMSCs (Figure 4.1D). The anti-

inflammatory effect was caspase dependent because it was reverted by generating ApoMSCs in the presence of Z-VAD-FMK (Figure 4.1E).

Figure 4.1. Apoptotic MSCs exert *in vivo* immunosuppression despite the absence of cytotoxic cells.

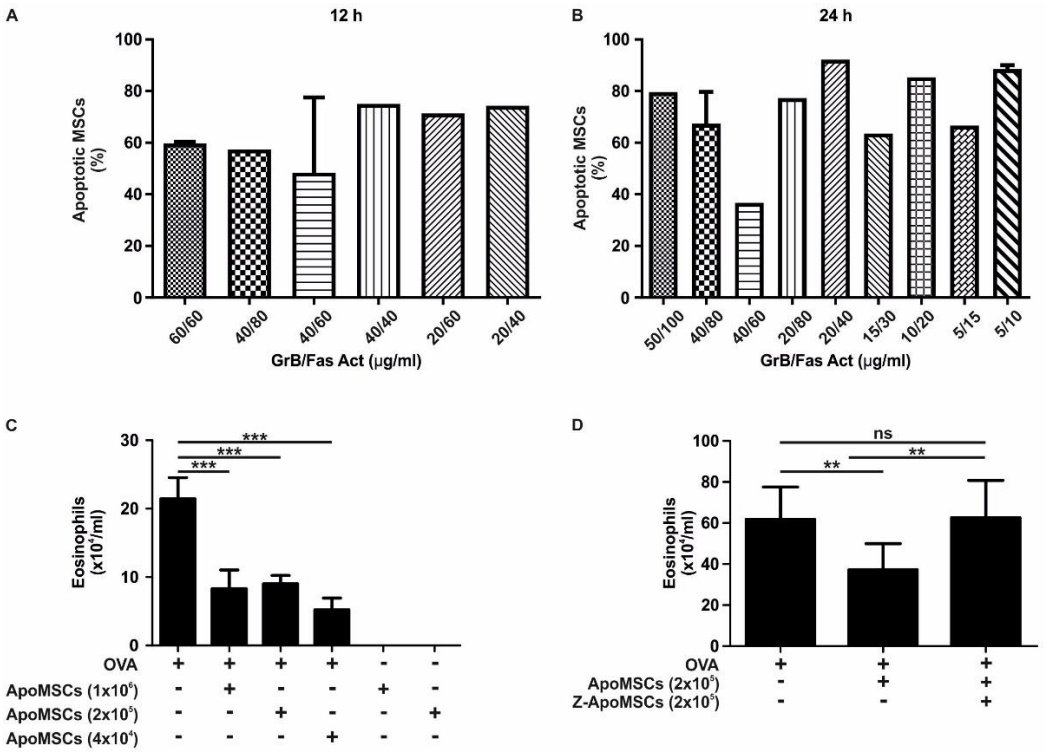

Figure 4.1. Apoptotic MSCs exert *in vivo* immunosuppression despite the absence of cytotoxic cells. A-B: MSCs were treated with different concentrations of recombinant human GrB and monoclonal antibody against human FAS (activating) in order to optimize the generation of *in vitro* apoptotic MSCs (ApoMSCs). Level of apoptosis was assessed by flow-cytometry after 12 (**A**) or 24 hours (**B**) of treatment. The most effective treatment selected was GrB/FAS (5/10 µg/ml) for 24 hours, which is able to generate a mean of 88.5% (SD: 1.56, 95% CI: 74.52-102.5) apoMSCs. **C:** Th2-induced airway inflammation was obtained in Balb/C mice as in Figure 3.17A. Where

indicated different doses of ApoMSC ($1x10^6$, $2x10^5$ and $4x10^4$) were injected 1 hour after the last challenge. Eosinophil infiltration in BAL was then evaluated after additional 18 hours. The results of three independent experiments are shown. Statistics: 1-way ANOVA and Hommel post-hoc test. **D:** Balb/C mice were treated as in C and injected 1 hour after the last challenge with ApoMSCs or ApoMSCs obtained adding the pan-caspase inhibitor Z-VAD-FMK during the 24 hours of incubation (Z-ApoMSCs). Eosinophil infiltration in BAL was evaluated after additional 18 hours and was $61.82x10^4$/ml (DS: 41.62, 95% CI: 23.33-100.30) in OVA only mice (n=7), $37.19x10^4$/ml (DS: 38.41, 95% CI: 7.65-66.71) in OVA mice treated with $2x10^5$ ApoMSCs (n=9) and $62.59x10^4$/ml (DS: 48.25, 95% CI: 17.96-107.2) in mice treated with $2x10^5$ Z-ApoMSCs (n=7). **C-D:** Results of three independent experiments are shown. Statistics: 1-way ANOVA and Hommel post-hoc test. **: p<.01, ***: p<.001. ns: not significant.

4.2.2 Apoptotic MSCs infused in GvHD are immunosuppressive and induce IDO production in recipient phagocytes.

We subsequently investigated whether ApoMSCs could be immunosuppressive also in the GvHD model. ApoMSCs were administered either i.v. or i.p. at day +1, +3, and +6 after the transplant, and the infiltration of $CD8^{+}V_{\beta}8.3^{+}$ Mh T cells was assessed and compared to untreated GvHD mice at day +7. ApoMSCs produced a substantial reduction in GvHD effector cell infiltration in both spleen and lungs. Mean percentage infiltration of Mh T cells was 7.58% (SD: 2.05, 95% CI: 5.86-9.29) and 11.72 (SD: 3.04, 95% CI: 9.55-13.89) in the spleen of ApoMSC treated and untreated GvHD mice, respectively (Figure 4.2A). In lungs the percentage of $CD8^{+}V_{\beta}8.3^{+}$ GvHD effector cells was 14.18% (SD: 7.83, 95% CI: 7.64-20.73) and 18.97% (SD: 6.95, 95% CI: 14.00-23.94) in treated and untreated mice, respectively (Figure 4.2B). Importantly, this immunosuppressive effect could not be observed in those mice treated with ApoMSCs infused i.v. (Figure 4.2C and D).

Figure 4.2. ApoMSCs exert immunosuppressive activity in GvHD.

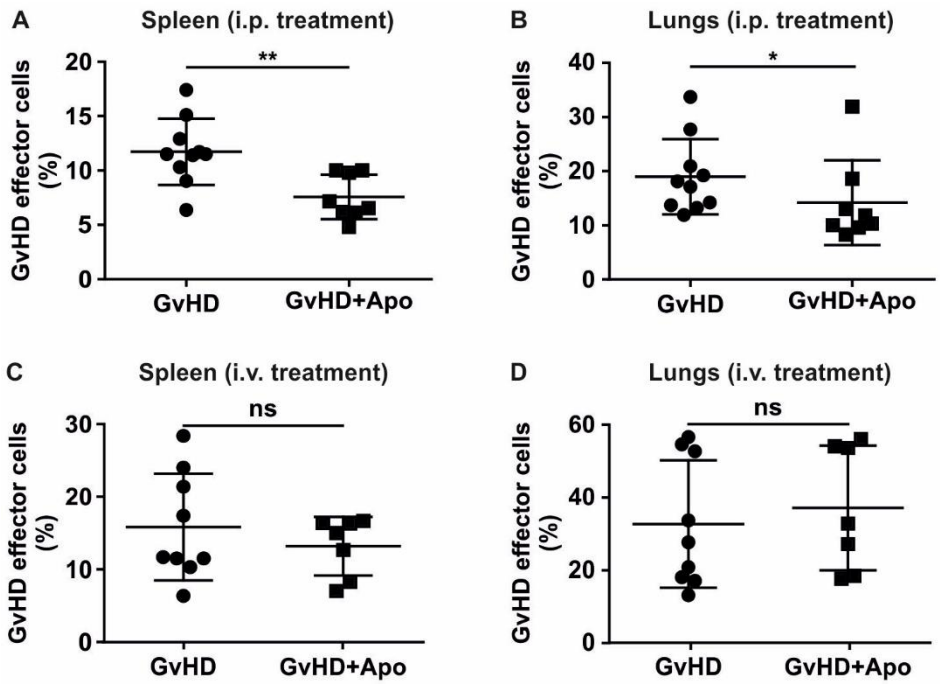

Figure 4.2. ApoMSCs exert immunosuppressive activity in GvHD. A-D: The percentage of GvHD effector cells was assessed in the lymphocyte gate in spleen (**A, C**) and lungs (**B, D**) of GvHD mice (black circles) and GvHD mice treated with ApoMSCs (black squares). ApoMSCs were infused i.p. (GvHD mice N=10, GvHD+ApoMSC mice N=8) (**A, B**), or i.v. (GvHD mice N=9, GvHD+ApoMSC mice N=7) (**C, D**). Results represent the mean±SD of 3 independent experiments. Statistics: unpaired t-test. *: p<.05; **: p<.01. ns: not significant.

It has been reported that the injection of irradiated thymocytes into animals results in their phagocytosis by recipient macrophages and induction of IDO[374]. We therefore tested whether ApoMSCs followed the same destiny by eliciting *in vivo* efferocytosis by recipient phagocytes and inducing IDO production. For this purpose, labelled ApoMSCs were traced in recipient phagocytes after injection. Following i.p. administration, ApoMSCs were largely identified inside $CD11b^+$ (Figure 4.3A) and $CD11c^+$ (Figure 4.3B) phagocytes in the peritoneal draining lymph nodes[421] but absent when searched for in the lungs and spleen. When the i.v. route was used, amongst the several phagocytic populations investigated[422], $CD11b^{high}CD11c^{int}$ (residual vessel patrolling monocytes/mature $CD11b^+$ cDCs), $CD11b^{high}CD11c^-$ (circulating monocytes) and $CD11b^-CD11c^+$ (DCs) were detected as engulfing ApoMSCs in lungs (Figure 4.3C-E). The analysis of IDO expression in the phagocytes engulfing ApoMSCs both in the i.v. and i.p. groups revealed that only the phagocytes in the i.p. group were able to increase IDO expression in comparison with their counterparts in untreated GvHD mice (Figure 4.3F and G). These findings strongly suggest that the immunosuppressive effect of ApoMSCs involves recipient phagocytes and IDO as crucial effector mechanisms.

Figure 4.3. ApoMSCs elicit IDO in engulfing recipient phagocytes.

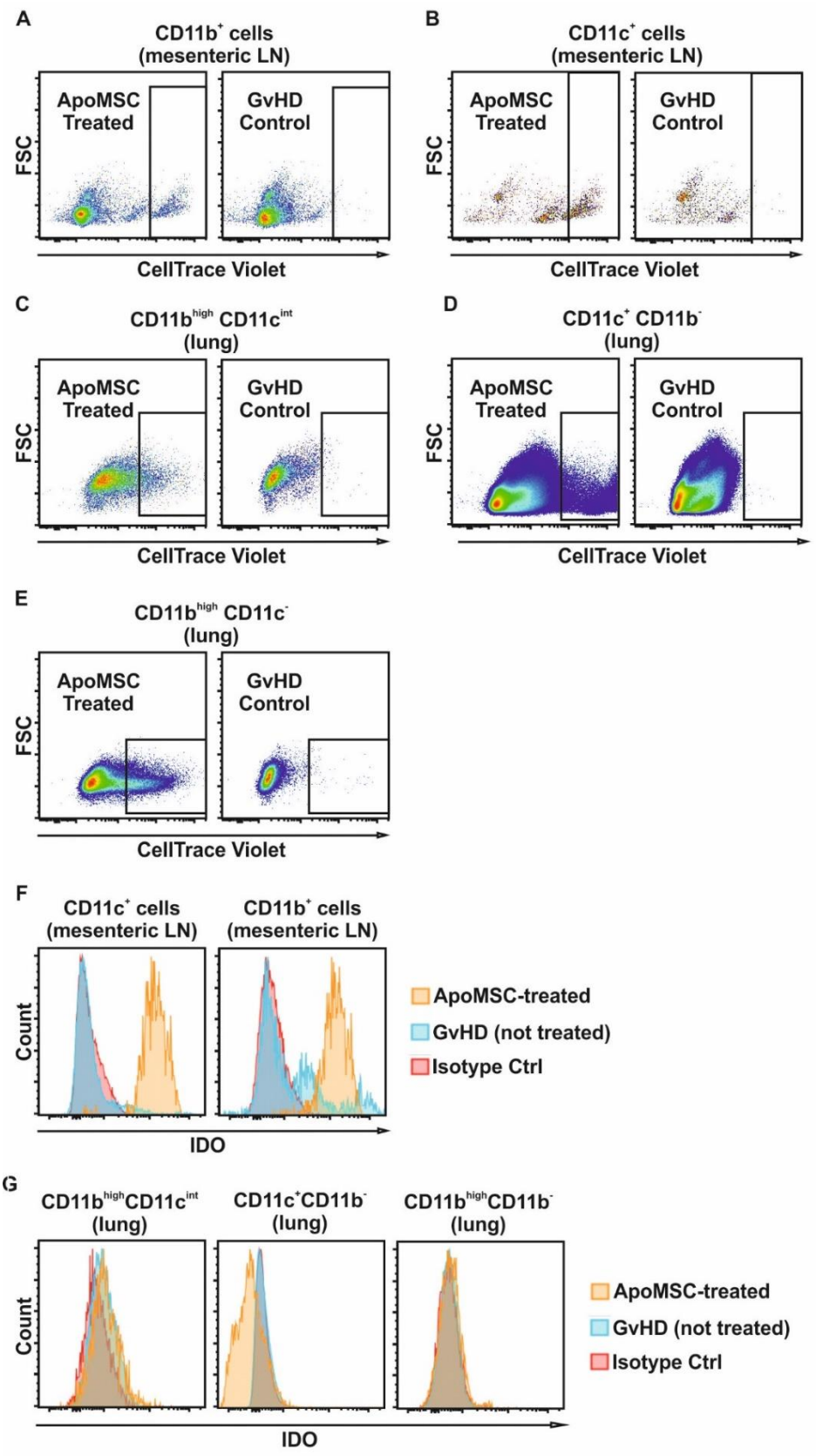

Figure 4.3. ApoMSCs elicit IDO in engulfing recipient phagocytes. A-G: MSCs were labelled using CellTrace™ Violet and subjected to apoptosis induction using GrB/FAS-L (5 µg/ml and 10 µg/ml, respectively). Labelled ApoMSCs were injected i.p. (**A**, **B** and **F**) or i.v. (**C**, **D**, **E** and **G**) into GvHD mice 3 days after the transplant. After 2 hours, animals were sacrificed and mesenteric lymph nodes (LN) (**A**, **B** and **F**) or lungs (**C**, **D**, **E** and **G**) were harvested. Cells engulfing ApoMSCs were identified as Violet^{+} cells within the CD11b^{+} (**A**), CD11c^{+} (**B**), CD11b^{high}CD11c^{int} (**C**), CD11c^{+}CD11b^{-} (**D**), and CD11b^{high}CD11c^{-} (**E**) subpopulations. The corresponding subpopulations were gated in GvHD mice which had not received violet labelled ApoMSCs and used as controls. **F** and **G**: IDO expression was assessed in CD11c^{+} and CD11b^{+} (**F**) or CD11b^{high}CD11c^{int}, CD11c^{+}CD11b^{-} and CD11b^{high}CD11c^{-} (**G**) cells positive for CellTrace^{TM} Violet (engulfing ApoMSCs) and compared with the corresponding populations in GvHD mice that had not received ApoMSCs. Data are representative of similar results obtained from three mice in 2 independent experiments.

4.2.3 Recipient IDO-producing phagocytes are indispensable for MSC immunosuppression in GvHD.

To directly test the importance of recipient-derived phagocytes and IDO activity in MSC immunosuppressive activity, we depleted phagocytes and inhibited IDO activity in GvHD mice before MSC treatment and evaluated the effect of live MSCs on the expansion of GvHD effectors. To deplete phagocytes, liposome clodronate was given to mice 72 hours before MSC injection[374]. The treatment substantially impaired the ability of MSCs to suppress Mh T cell infiltration (Figure 4.4A and B).

Finally, animals were given the IDO inhibitor 1-methyl-D-tryptophan (1-DMT)[375] before MSC injection. Also in this case, the beneficial effect of MSCs on Mh T cell infiltration was much reduced in mice receiving 1-DMT compared to controls (Figure 4.4C and D). However, these results do not definitely exclude that IDO could be produced, at least in part, by the apoptotic MSCs. If this was the case, also ApoMSCs by themselves could have mediated the generation of tolerogenic phagocytic cells. To address this question, we evaluated the *IDO* gene expression in ApoMSCs by real time PCR after 12 and 24 hours of incubation with GrB and FAS. Our findings demonstrated that in ApoMSCs IDO is not induced during the apoptotic process (Figure 4.4E and F).

We therefore conclude that the immunosuppressive effect of MSCs requires the presence of recipient phagocytic cells in the host which eventually produce IDO.

Figure 4.4. Recipient phagocytes and IDO production are required for MSC immunosuppressive activity in GvHD.

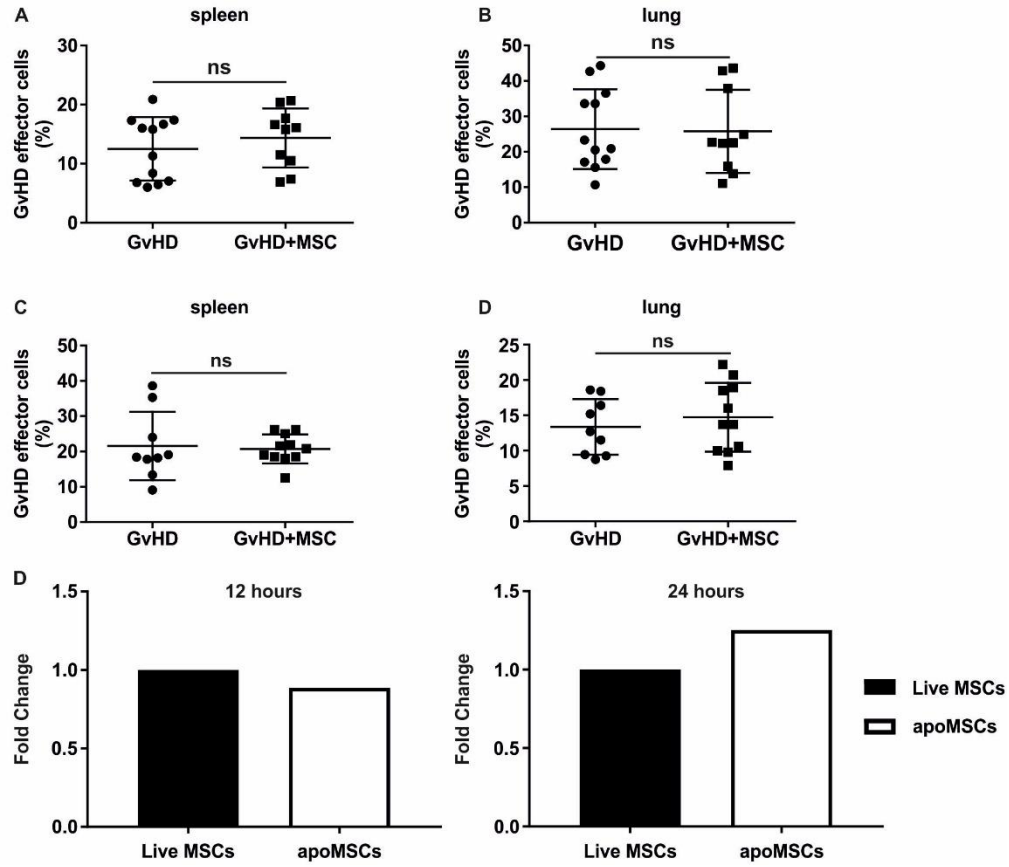

Figure 4.4. Recipient phagocytes and IDO production are required for MSC immunosuppressive activity in GvHD. A, B: GvHD mice were treated with liposomal clodronate 10 minutes after the transplant. Where indicated, MSCs were infused 3 days later. The percentage of GvHD effector cells ($CD8^+V_\beta8.3^+$) were calculated in the lymphocyte gate in spleen (**A**) or lungs (**B**) after 4 additional days. Mean±SD was obtained grouping three independent experiments with N: 12 (GvHD) and 10 (GvHD+MSCs) mice per group. **C, D:** GvHD effector cell infiltration was studied in spleen (**C**) and lungs

(**D**) of GvHD mice treated with the IDO-inhibitor 1-DMT. In the treated mice, MSCs were infused 3 days after the transplant (N=11). Controls consisted of GvHD mice which did not receive MSCs (N=9). Percentage of $CD8^{+}V_{\beta}8.3^{+}$ cells refers to the lymphocyte population. Results refer to the mean±SD of 3 independent experiments. Statistics: unpaired t-test. *: p<.05; **: p<.01. ns: not significant. **D:** MSCs were incubated for 12 or 24 hours with GrB (5 µg/ml) and activating FAS (10 µg/ml) to generate ApoMSCs. *IDO* expression was assessed by real time PCR and calculated as relative expression in comparison to that of untreated (live) MSCs. Representative results of two independent experiments are shown.

4.2.4 Recipient-derived IDO is indispensable for ApoMSC immunosuppression in a Th2-type inflammation model.

To assess that also the immunosuppressive activity delivered by ApoMSCs was dependent on IDO produced by MSC-recipients, OVA-treated animals were given the IDO inhibitor 1-DMT[375] before ApoMSC injection. As in the GvHD model, the beneficial effect of ApoMSCs on eosinophil infiltration was abolished in mice receiving 1-DMT. Hence, also in the case of MSCs made apoptotic *in vitro*, their anti-inflammatory effect requires IDO production by the host (Figure 4.5). We therefore conclude that the anti-inflammatory effect of ApoMSCs generated *ex vivo* requires the presence of IDO in the host receiving MSCs also in this model.

Figure 4.5. IDO production by apoMSC recipient is required for immunosuppression in a Th2-type inflammation model.

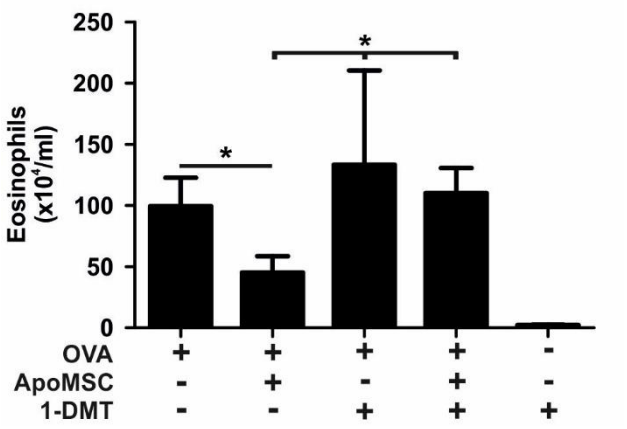

Figure 4.5. IDO production by apoMSC recipient is required for immunosuppression in a Th2-type inflammation model. Balb/C mice were treated as in Figure 20A and injected with ApoMSCs ($2x10^5$) 1 hour after the last challenge. Eosinophil infiltration in BAL was then evaluated after additional 18 hours. Where indicated mice were treated with 1-DMT in the drinking water (2 mg/ml) starting 6 days before MSC injection. Eosinophil infiltration was 99.42x104/ml (DS: 57.47, 95% CI: 39.11-159.70) in OVA only mice (n=6), 45.24x104/ml (DS: 32.98, 95% CI: 10.63-79.85) in OVA mice treated with ApoMSCs (n=6), 133.5x104/ml (DS: 76.95, 95% CI: 37.96-229.1) in OVA mice treated 1-DMT (n=5), 110.3x104/ml (DS: 54.28, 95% CI: 60.06-160.5) in OVA mice treated with ApoMSCs and 1-DMT (n=7) and 2.31x104/ml (DS: 0.39, 95% CI: 1.32-3.30) in naïve mice treated with 1-DMT (n=3). Results of two independent experiments are shown. Statistics: 1-way ANOVA and Hommel post-hoc test.

4.3 Discussion

In this Chapter we have shown that MSCs made apoptotic *in vitro* exert potent immunosuppression in both a preclinical model of aGvHD and in a Th2-type inflammation model of lungs. These regulatory functions are mediated through the production of IDO by the phagocytic cells engulfing ApoMSCs.

Our data further support an approach to MSC therapeutics that highlights the key role of MSC recipient to orchestrate and determine MSC effector functions as discussed in the previous Chapter. Not only are cytotoxic cells in the recipient required to initiate apoptosis in infused MSCs (Chapter 3), but also phagocytes which, by engulfing apoptotic MSC and producing IDO, ultimately deliver MSC immunosuppressive activity.

The link between phagocytosis of apoptotic MSCs, induction of tolerogenic phagocytes and IDO production has important implications in the design of future clinical trials employing MSCs. The monitoring of tolerogenic phagocytic populations of cells may be devised as a biomarker of response after treatment, as also suggested by the observation of a more marked increase of circulating tolerogenic DCs in aGvHD patients who responded to MSCs in comparison with non-responding patients[297]. Furthermore, the possibility to assess kynurenines in serum samples[423], or to track IDO activity *in vivo* with analogues of Tryptophan which can be imaged with positron emission tomography[424], might open the way to the development of new

surrogates of outcomes based on IDO activation. Although still at a very early stage, these new hints warrant further investigations.

The use of cells made apoptotic before infusion is an intriguing approach which have been attracting much attention in solid and BM transplant management[407]. Also in GvHD, the use of patient white blood cells made apoptotic after exposure to photoactivatable 8-methoxypsoralen and ultraviolet A radiation (extracorporeal phonophoresis [ECP]) is a consolidated, although not standardised, therapy in steroid resistant patients[425,426].

The mechanisms unveiled in this chapter are in line with those reported to explain how apoptotic cells of different lineages, generated *in vitro*, induce immune modulation in GvHD[406–408], and macrophage IDO production in other systemic autoimmune diseases[375].

The depletion of recipient macrophages or the inhibition of IDO activity also impairs the therapeutic activity of live MSCs, thereby linking *in vivo* MSC apoptosis with immunosuppression. It is unlikely that any particular phagocyte population (macrophages or dendritic cells) is selectively involved in engulfing ApoMSCs because they similarly display such an activity *in vivo*.

The crucial role advocated for phagocytic cells as ultimate effectors of the immunosuppressive tolerogenic effect mediated by MSCs is consistent with the described ability of MSCs to stimulate recipient immune tolerance networks, macrophages and DCs (Chapter 1). In this study we have not analysed the role of Treg cells. This would have required an analysis taking into account the kinetics of Treg cell generation as well as their localisation and trafficking. Furthermore, recipient myeloablation could be a confounding factor because it is associated with a homeostatic expansion that has been reported to selectively prime Treg cell expansion[427,428].

However, it is conceivable that stimulation of IDO production in macrophages and DCs after engulfment of apoptotic MSCs (generated *in vivo* or *in vitro*) may be one of the mechanisms employed for the generation of Treg cells *in vivo* after MSC infusion, since IDO is implicated in the expansion of regulatory T cells (Chapter 1).

Another important impact of this study is that the administration of *ex-vivo* generated ApoMSCs can circumvent the requirement for cytotoxic cells in a Th-2 type inflammatory model. The fact that ApoMSCs can be effective at suppressing the expansion/infiltration of the GvHD effector cells supports the idea that this therapeutic tool may be exploited to treat those aGvHD patients who do not respond to MSCs because deficient in those cytotoxic cells able to induce MSC apoptosis. Interestingly, ApoMSCs were mostly effective in the GvHD model only when administered i.p. Despite being phagocytosed,

ApoMSCs injected i.v. did not induce IDO production, thus suggesting that the site at which MSC apoptosis occurs may influence the immunosuppressive function, perhaps by engaging with a subpopulation of phagocytes. Therefore, a more thorough characterization of the administration modality is required before testing ApoMSCs in the clinical setting.

Our data indicate that MSC apoptosis is sufficient to deliver a therapeutic activity in two different disease models, thus indicating that, at least in our experimental conditions, the cytokine-mediated 'licensing' (see Chapter 1) is not required for MSC immunosuppression. However, we cannot exclude that, before undergoing apoptosis, MSCs directly inhibit inflammatory reactions through the conventional pathways. Furthermore, caspase activation in MSCs may trigger cell-death independent pathways that stimulate the synthesis of immunomodulatory molecules independently of the generation of signals for phagocytosis[429]. Consistent with this, it has been shown that MSCs activate caspase-dependent IL-1 signaling that enhances secretion of immunomodulatory molecules[136].

Our data on the role of MSC apoptosis and the possibility to employ MSCs already made apoptotic *in vitro* before infusion open important questions which may be clinically relevant in specific settings and thus need to be addressed in future studies. One first question is whether the antigen repertoire of MSCs could have a role in mediate antigen specific tolerance.

This issue may be relevant when MSCs are used for the prevention or treatment of solid organ transplant rejection. Indeed, it has been reported in a mouse model of hearth transplantation that prolonged survival of the graft could be obtained only when MSCs and tolerized grafts shared part of their antigens, but not when they were completely disparate[237].

The final question is whether MSCs, once killed, may elicit an allo-specific immune response which may affect subsequent infusions of MSCs from the same donor. It has been demonstrated that MSCs rejected in allogeneic settings elicit an immune response with induction of memory cells both within the $CD4^+$ and $CD8^+$ compartments[401]. These memory cells were able to rapidly lysed MSCs upon re-infusion, and the killing was alloantigen-specific. If this will be confirmed also in the GvHD settings, it would support the usefulness of sequential infusion of MSCs, ideally from the same donor, whereby the previous infusion would boost the response to the next administration of cells. However, the usually severely immunocompromised status of the patient may be an obstacle to this strategy.

5 Response to MSCs within the first week after infusion significantly improve the survival of GvHD patients.

5.1 Introduction

In the previous Chapters we have demonstrated that MSCs undergo apoptosis after infusion. MSC apoptosis is instrumental for the therapeutic activity and requires the presence of cytotoxic cells in MSC-recipient. Deciphering this novel mechanism of action led us to the identification of a biomarker to predict clinical responses in GvHD patients treated with MSCs. Importantly, our functional assay was the only factor associated with a response to MSCs.

Our responses were recorded as early as one week after MSC treatment. The choice of an early assessment was in line with our hypothesis that crucial events leading to the immunosuppression delivered by MSCs could take place early after infusion. Furthermore, it minimizes the risk of possible confounding effects of concomitant treatments on the response to MSCs. However, the clinical significance of an early assessment remains to be addressed, since the current practice to evaluate responses to MSC treatment has been 28 day time-point[338,430].

In this Chapter, we first analyzed the overall survival of the 32 patients enrolled in the study of the cytotoxic assay and described in Chapter 3, and we assessed whether evaluation of the response at 1 week had a significant impact on the survival of those patients. To confirm the clinical relevance of this early assessment, we performed a retrospective analysis of a cohort of 60 steroid-resistant aGvHD patients treated with BM-MSCs in several centres of the UK, one of the largest series of patients treated with MSCs reported so far.

5.2 Results

5.2.1 Impact of assessing clinical response at 1 week after MSC treatment.

To investigate the significance of assessing MSC treatment at 1 week, we evaluated the overall survival of the 32 patients who were enrolled in the study of the cytotoxic assay, described in Chapter 3. Patients characteristics are described in Table 3.1.

The median OS of the whole cohort of patients was 72 days (95% CI: 78.06-250.7 days) from GvHD diagnosis (Figure 5.1A), with a median follow-up of 480 days for patients alive (range 91-592 days). Three patients (2 among the non-responders and 1 among responders) died because of relapse soon after MSC infusion and were excluded from the study. When patients were classified into responders and non-responders to MSCs, we could find a significant longer overall survival in those patients classified as responders. Median survival was 65 days or not reached in non-responders and responders, respectively (Figure 5.2B).

These data demonstrated that assessment of the response at 1 week from MSC treatment did have clinical relevance.

Figure 5.1. Overall survival of patients treated with MSCs.

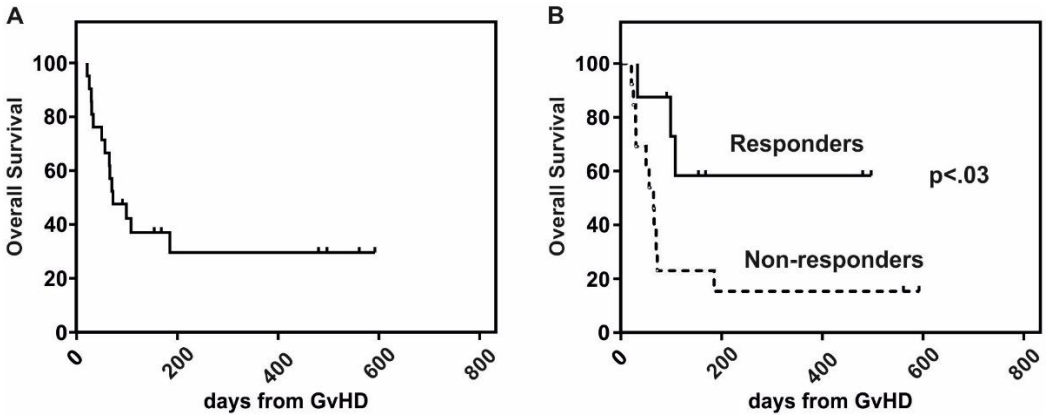

Figure 5.1. Overall survival of patients treated with MSCs. A: OS of the whole cohort of patients. **B**: OS of Responders and Non-responders. log-rank test, p<.02; hazard ratio: 0.27 (95% CI: of ratio 0.096 to 0.784). Median survival in Responders was not reached, while was 65 days in Non-responders. Overall survival starts from GvHD diagnosis.

5.2.2 Retrospective analysis of a cohort of 60 patients with steroid resistant GvHD treated with MSCs.

Sixty steroid-resistant aGvHD patients treated with BM-MSCs at several Centres in the UK between May 2008 and December 2014 were retrospectively studied. Detailed demographics of patients are summarized in Table 5.1.

Median age of patients was 40 years (4 months-68 years). Most of the patients were transplanted because of a haematological malignancy. Fourteen patients had AML, 10 patients had ALL, 10 had CML, 7 had MDS or MPNs, 11 patients were diagnosed with HL, NHL, or MM and 11 had other non-neoplastic diseases.

All patients received GvHD prophylaxis with 3 or 4 doses of methotrexate combined with CSA, and T-cell depletion with alemtuzumab or ATG. aGvHD was biopsy proven in 46 (77%) patients, whilst in the remaining patients the diagnosis was based on clinical features after excluding alternative causes.

Fifty-five (92%) patients had grade III or IV GvHD. Ten (17%), 16 (27%), and 1 (2%) of patients had skin, gut, or liver involvement only, respectively. 16 (27%) patients exhibited gut and skin, 11 (18%) skin, gut, and liver, 3 (5%) skin and liver, and 3 (5%) gut and liver.

Most of the patients of this cohort were heavily pre-treated. Indeed, 47 (78%) received MSCs after two or more therapies. When other drugs were used after steroids, the most used were MMF (n=30, 50%), monoclonal antibodies against TNFα (n=18, 30%), or CSA (n=17, 28%). Only in 13 (22%) patients, MSCs were given as second line treatment after methylprednisolone.

Table 5.1. Patients' characteristics.

Total, number	60
Age, years	
Median (Range)	40 (4mo-68)
Sex, n	
Male	21
Female	39
Disease, n	
AML	14
ALL	10
CML	10
MDS/MPNs	7
CLL	1
NHL/HL/MM/Other Lymphomas	11
Others	7
Time from HSCT to MSC treatment, days	
Median (range)	62 (12-929)
Time from aGvHD to MSC treatment, days	
Median (range)	60 (11-905)
GvHD treatment before MSC infusion, n	
MEP Alone	13
MEP in combination with other drugs	45
Other drug combinations not including MEP	2
CSA	17
MMF	30
Anti-TNFα (Infliximab, Etanercept)	18
Tacrolimus	8
MTX	3
Anti-CD25 (Basiliximab, Daclizumab)	1
ECP	3
Anti-CD20 (Rituximab)	1
Anti-CD52 (Alemtuzumab)	3
ATG	1
GvHD grade, number	
I-II	5
III-IV	55
Biopsy Proven, number	
Yes	46
No	14

aGvHD: acute Graft versus Host disease, **AML:** Acute Myeloid Leukemia, **ALL:** Acute Lymphoblastic Leukemia, **ATG:** anti-thymocyte globulin, **CML:** Chronic Myeloid Leukemia, **CLL:** Chronic Lymphocytic Leukemia, **CSA:** Cyclosporin, **ECP:** Extracorporeal photopheresis**, HSCT:** Hematopoietic Stem Cell Transplant, **MDS/MPN:** Myelodisplastic Syndrome/Myeloproliferative Neoplasms, **MEP:** Methylprednisolone, **MMF:** Mycophenolate, **MSC:** Mesenchymal Stromal Cells, **MTX:** Methotrexate**, NHL/HL/MM:** Non-Hodgkin Lymphoma/Hodgkin Lymphoma/Multiple Myeloma, **TNFα:** Tumor Necrosis Factor α.

5.2.3 MSC treatment.

Thirty-four patients (57%) received 1 dose of MSCs, while 19 (32%), 6 (10%) and 1 (2%) were treated with 2, 3, or 4 doses, respectively. Median time to first MSC administration was 62 days (12-929 days) from HSCT or 60 days (11-905 days) from GvHD diagnosis. The median dose of MSCs was $2.6x10^6$/Kg body weight per infusion (range: 0.6-$15.6x10^6$/Kg body weight). No significant adverse reactions were observed after infusion.

5.2.4 Response to MSC treatment.

Response to MSCs was assessed 1 week after treatment. Overall, 32 patients (53%) responded, with 1 (1.6%) and 31 (51.6%) patients achieving CR or PR, respectively. Amongst patients who received multiples doses, in most cases subsequent doses did not change the type of response obtained after the first dose, except for two patients. One patient responded to the first dose with a PR, received a second one but relapsed a week after. The second patient achieved PR after the first dose and CR after the second.

We then investigated whether we could identify factors associated with clinical responses. We found that patient gender, pre-MSC therapy, interval from HSCT or aGvHD diagnosis to MSC treatment, and grade of aGvHD did not affect clinical responses (Table 5.2). Conversely, organ involvement, age at HSCT and the dose of MSCs were significantly associated with the response rate to MSCs (Table 5.2). The proportion of responders was 28 (67%) amongst patients with involvement of gut, skin, or both, but only 4 (22%) amongst those with involvement of the liver (alone, or in combination with skin and/or gut). Patients younger than 20 years fared better, with 15 (88%) of them responding to MSCs. Conversely, only 7 (30%) and 6 (43%) of those aged 20-50 years or older than 50 responded, respectively. Lastly, higher response rates (n=13, 77%) were observed in patients receiving MSC doses >3.0x106/Kg compared with patients receiving 1.5-3.0x10^6/Kg (n=18,

56%) or <1.5x10^6/Kg (n=1, 9%) (Table 5.2). All these 3 factors remained significant in multivariate logistic regression analysis (Table 5.3).

Table 5.2. Analysis of factors affecting survival and response to treatment.

	N	Median Survival, months (95%CI)	*P*	Response, %	*P*
Overall, number	60	3.4 (0-7.8)	-	32 (53%)	-
Patient Gender, number					
Male	21	2.6 (0-7.3)	*0.23*	12 (57%)	*0.66*
Female	39	7.7 (0-27.1)		20 (51%)	
Patient age, years					
<20	17	16.3 (NC)		15 (88%)	
20-50	23	1.3 (0-7.1)	*0.11*	7 (30%)	*0.008*
>50	14	1.7 (0.5–2.8)		6 (43%)	
aGvHD treatment, number					
MEP alone	12	4.8 (0-27)		4 (33%)	
MEP +1 other	13	5.0 (0.6-9.5)	*0.96*	8 (61%)	*0.14*
MEP +2 or more	30	2.1 (0-5.8)		20 (67%)	
Time from HSCT to MSCs, days					
<60	26	1.9 (0-4.3)	*0.23*	14 (54%)	*0.78*
>59	28	16.3 (0-39)		14 (50%)	

Time from GvHD to MSCs, days <60 >59	28 22	3.1 (0-6.7) 1.7 (0-5.0)	*0.82*	16 (57%) 8 (36%)	*0.14*
aGvHD grade, number 2 3 4	5 25 29	22.9 (0-37) 2.3(0-9.0) 2.7 (0-5.5)	*0.46*	1 (20%) 15 (60%) 16 (55%)	*0.42*
aGvHD organ Skin or Gut or Skin + Gut Other	42 18	16.3 (0-37.0) 0.6 (0.3-1.0)	*0.008*	28 (67%) 4 (22%)	*0.002*
MSC dose, x10^6/Kg body-weight <1.5 1.5-3.0 >3.0	11 32 17	2.2 (0-4.7) 3.9 (0-11.9) 5.0 (0-10.1)	*0.62*	1 (9%) 18 (56%) 13 (77%)	*0.001*
Response to MSCs Yes No	32 28	Not achieved 0.6 (0.4-1.0)	*<0.001*		

aGvHD: acute Graft versus Host disease, **HSCT**: Hematopoietic Stem Cell Transplant**, MEP:** Methylprednisolone, **MSCs:** Mesenchymal Stromal Cells.

Table 5.3. Multivariate logistic regression analysis for disease response.

	N	Odds ratio (95%CI)	*P*
Patient age, years			
<20	**17**	**1.00**	
20-50	**23**	**0.10 (0.02-0.72)**	***0.022***
>50	**14**	**0.45 (0.05-4.66)**	***0.46***
aGvHD organ, number			
Skin or Gut or Skin + Gut	**42**	**1.00**	
Other	**18**	**0.10 (0.01-0.78)**	***0.028***
MSC dose, $x10^6$/Kg body-weight			
<1.5	11	1.00	
1.5-3.0	32	6.90 (0.55-86.50)	*0.14*
>3.0	17	28.22 (1.70-477.04)	*0.021*

aGvHD: acute Graft versus Host disease, **MSCs:** Mesenchymal stromal Cells.

5.2.5 Analysis of Survival

The estimated median OS of all patients was 104 days (95% CI: 0-215 days) (Figure 5.2A), with a median follow-up of 741 days for patients alive (range 461–2521 days). Response to MSCs had a major impact on OS, with a longer estimated OS in responding patients compared with non-responders (Figure 5.2B).

We evaluated the association between OS and gender, age, pre-MSC therapy, interval from HSCT, or aGvHD diagnosis to MSC treatment, grade of aGvHD, organ involved, and the response to MSCs. By using both univariate and multivariate analysis, we found that the presence of any kind of response (CR or PR) assessed after 1 week from the first MSC infusion and the organ affected by GvHD were strong predictors for survival (Table 5.2).

Figure 5.2.Probability of survival in patients treated with MSCs.

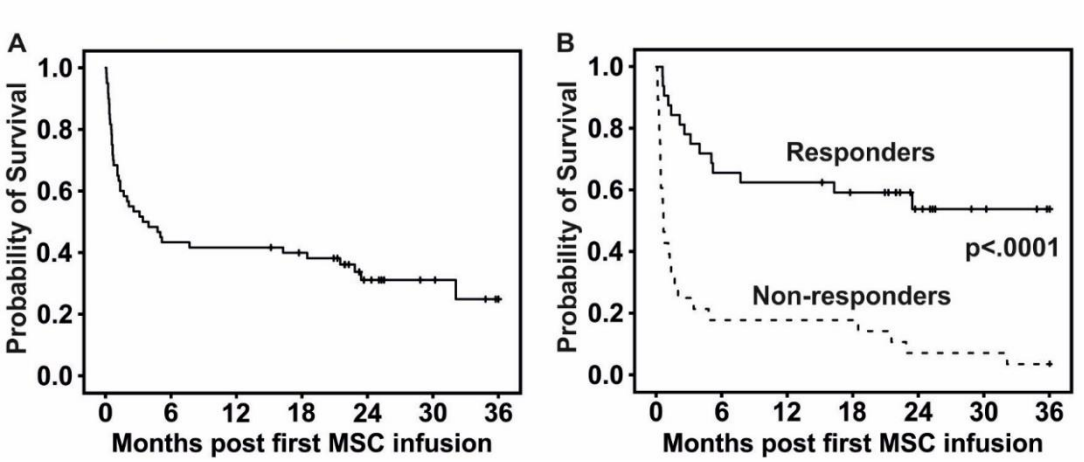

Figure 5.2. Probability of survival in patients treated with MSCs. A: Probability of Survival of the whole cohort of patients. **B**: Probability of Survival of Responders and Non-responders. log-rank test, p<.0001; hazard ratio: 0.2. Median survival in Responders was not reached, while was 20 days (95% CI 11–29 days) in Non-responders. Overall survival estimates start from MSC infusion.

5.3 Discussion

The data described in this chapter strongly support the clinical relevance of assessing response 1 week after MSC administration. Responders defined at this time-point exhibited a much longer overall survival in comparison with non-responders. Our observation is reinforced by the fact that this finding was confirmed in cohorts of patients from different Centres. The clinical relevance of the response at 1 week strengthens the importance of our cytotoxic assay described in Chapter 3, since this biomarker is predictive of an early response. We anticipate that, once validated in larger cohort of patients, this biomarker could be devised to predict patients' long-term outcome.

Our observation has also other important implications. In current practice, response to MSC treatment has been assessed after 28 days[338,430], based on previous recommendations that 28 days would represent the most reliable time point to predict survival of GvHD patients[431]. However, it is not clear whether an early time point can be used to reliably categorize patients at high risk of treatment failure or who may benefit from starting alternative therapeutic options. The importance of this problem has been already the focus of intense debate in GvHD patients (although not referred specifically to MSC treatment) and several studies have identified earlier endpoints as predictors of later outcomes[432,433]. Our data demonstrate that clinical responses at 1 week after MSC infusion can be considered an early predictor of treatment failure. An early assessment has therefore the advantage to

provide crucial information to promptly offer alternative approaches both in clinical practice and in the context of clinical trials.

This approach might underestimate the rate and magnitude of clinical responses. In fact, the rate of CR in our cohorts is very low compared to other studies[329,342]. In our study, we could not document whether our responding patients eventually achieved CR at later time points, because we were unable to retrieve consistent data after day 7. However, the obtainment of any kind of response, either partial or complete, was sufficient to affect OS. This is consistent with previous studies in patients who, like ours, were refractory to several lines of treatment[331,337–339,345,430].

We also found that MSC dose, age of MSC-recipients and GvHD organ involvement are all factors affecting the response to MSCs. Because of the retrospective nature of the study, the dose range of MSCs was large thereby allowing us to identify a significant association between higher doses and a positive response. This observation is in contrast with other reports[329,330,338], but the discrepancy could be ascribed to the fact that in those studies the dose ranges were too narrow and the number of patients too small to identify any correlation between response and dose. Indeed, our data have been confirmed by a recent multicenter prospective study, in which patients who received doses of 3-4x10^6/Kg body weight had better responses and longer survival rates than those who received 1-2x10^6/Kg body weight[339].

Our study confirms that patient age and the affected organ significantly affect responses to MSCs[329,335,341,342,430]. In contrast, we could not find any association between GvHD grade[242,341,430], time from GvHD[330] or from HSCT[430] to MSC treatment and response to MSCs.

In summary, our data strengthen the role of the MSC recipient rather than the one of MSC donor or source in predicting clinical responses. This observation is supported by the findings of other groups[297,327,329,330,338], whereby when MSCs from the same donor were used to treat several patients, only a proportion of them achieved a response. Such a perspective suggests that the variability in MSC manufacturing bears a limited impact[371]. Overall, these findings strengthen the experimental data and the vision described in the previous Chapters and provide an innovative angle to improve the design of future clinical studies for the treatment of GvHD with MSCs

6 Conclusions and Future directions

MSCs have received centre stage attention because they exhibit potent immunosuppressive and anti-inflammatory activities[1,33] that have been extensively tested in several medical conditions, ranging from autoimmune diseases to the immunological complications of clinical transplantation, including GvHD. The extensive clinical use has been undeterred by the fact that the mechanisms underlying MSC therapeutic activity remain largely unresolved. The design of studies to convincingly assess MSC efficacy has been undermined by a few unresolved challenges. The first is the lack of early predictors of clinical responses, which severely delays the assessment of treatment success or failure. The second is that, only a proportion of patients responds to MSC infusions, and this response cannot be predicted. The third is that MSCs are not required to engraft to be efficacious.

In our study we addressed these questions, and we demonstrated that assessment of the response at 1 week is a reliable predictor of treatment failure. Furthermore, as summarized in Figure 6.1, by tracking the fate of MSCs after infusion, we demonstrated that MSCs are induced to undergo apoptosis by the cytotoxic cells of the recipient and this is instrumental for MSC therapeutic activity. Indeed, immunosuppression could be elicited only after IDO production induced in phagocytic cells after efferocytosis of the apoptotic MSCs.

These findings represent a paradigm shift in MSC therapeutics because we resolved the paradox of the presence of clinical activity in the absence of any engraftment. A further impact of our discovery is that the principle underpinning this mechanism could be used as a biomarker to predict clinical responses to MSCs and therefore stratify GvHD patients for MSC treatment.

Figure 6.1. MSC immunomodulation depends on the interaction with the host.

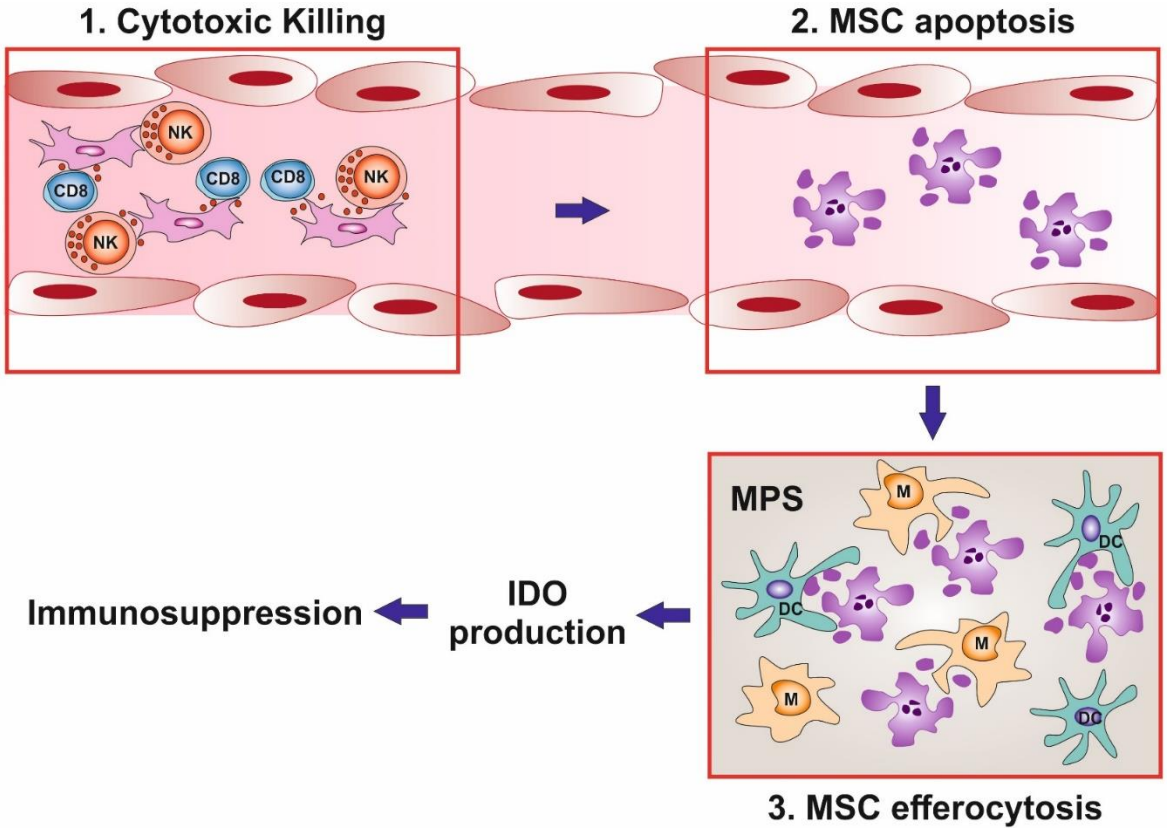

Figure 6.1. MSC immunomodulation depends on the interaction with the host. Schematic representation of MSC mediated immunosuppression after infusion. **1:** After infusion, MSCs interact with the cytotoxic granules produced by $CD8^+$ cells and NK cells of MSC recipient. **2:** MSCs are induced to undergo apoptosis. **3:** apoptotic MSCs are cleared from the circulation by the Mononuclear Phagocyte System (MPS). After efferocytosis, phagocytic cells of MSC recipient are induced to produce IDO which is the final mediator of MSC immunosuppression.

The role played by the cytotoxic cells and the production of IDO by host phagocytes, along with MSC-recipient age and GvHD organ involvement, strengthen patient's criticality for the achievement of a clinical response to MSCs. We therefore believe that the next generation of clinical trials should shift from choosing the best MSC population to choosing the patients most likely to respond. However, the cytotoxic assay that we propose to stratify patients for treatment can also be devised as a tool to standardize MSC manufacturing, and provide a new release criterion for regulatory authorities[434] and fulfilment of industrial demands[435] because it can support harmonization across the MSC products as an immunosuppressive agents.

The intriguing possibility that ApoMSCs may be effective in patients otherwise refractory to "conventional" "alive" MSCs paves the way to new avenues in the clinical manufacturing of MSCs.

Our data also open new important questions which yet remain to be evaluated in future studies. Whether and how the cytotoxic activity against MSCs is linked to recipient age and GvHD organ involvement, factors associated to response[329,335,341,342,430], need further investigations in larger studies. An important issue is whether children bear a stronger cytotoxic signature than older patients. Conversely, in the case the cytotoxicity is the same, the impact of the age on response can be mainly dependent on the different therapeutic approaches between the two groups, considering that children are usually treated early after steroid failure. This consideration raises another important

question about the possible detrimental effects of concomitant or previous therapies on the capacity of patients to induce MSC apoptosis or tolerogenic phagocytic cells after the uptake of the apoptotic bodies.

Another important question is how MSC apoptosis can be placed in the armamentarium of MSC immunosuppressive properties. It is likely that the signaling pathways associated with caspase activation and apoptosis prompt MSCs to directly produce immunosuppressive molecules, or interactions with other cells that may strengthen the final immunosuppressive effect and promote a long-term tolerogenic activity, as suggested by clinical data. Indeed. we have learned that it is the early evidence of a clinical response to be sufficient to predict patients' overall survival. This finding strongly suggests that MSCs are instrumental at re-educating the recipient immune system rather than simply suppressing an exuberant immune response.

This dissertation provides an explanation of some of the discrepancies that have undermined our understanding of MSC immunobiology. However, we are only scratching the surface of the challenge in our attempt to improve the use of MSCs in GvHD and other inflammatory diseases. New questions need to be addressed and new paths identified to pave the way.

This study has not addressed the question why ApoMSCs exerted an immunosuppressive activity which seems to be less potent than that of MSCs

when administered alive. Since we have not studied whether a cytokine-dependent licensing coexists with the generation of apoptotic MSCs, we cannot exclude that, before undergoing apoptosis, MSCs directly inhibit inflammatory reactions through the conventional pathways.

It is possible that before dying, MSCs are still capable to generate an immunosuppressive niche by means of soluble factors or interaction with other cells. Thus, it can be speculated that, to generate their maximal therapeutic potential, MSCs need first to home to the site of injury or inflammation, and only when there they need to be induced to undergo apoptosis. A very attractive hypothesis could be to genetically engineer MSCs with the ability to self-activate apoptosis upon activation of an inducible suicide gene, independently of the recipient capacity to kill MSCs. This would maximise MSC therapeutic activity in non-responsive patients by delivering immunosuppression “on demand” when MSCs home to the target organs, whereby they can exert immunosuppression by both the conventional and the apoptotic pathways.

A follow-on question regards the extent and the durability of the tolerogenic environment created by apoptotic MSCs. The restricted location of MSC apoptosis does not seem to reconcile with the systemic effects on inflammation. However, the tolerogenic phagocytes generated as a consequence of efferocytosis of apoptotic MSCs are in principle conveyors of infectious tolerance, since it has been demonstrated that IDO production by

macrophages engulfing apoptotic cells initiate the induction of an immunosuppressive phenotype of neighboring cells[374,375,436].

“The beginning of knowledge is the discovery of something we do not understand” (Frank Herbert). The concept of the need for MSCs to be killed after administration may be a beginning of a new fascinating era of this potent, yet difficult to manage, therapeutic tool.

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